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15348

Toxicity of Solutions of Cocaine Hydrochloride of Various Ages.

DAVID T. CARR AND HIRAM E. ESSEX.
From the Division of Experimental Medicine, Mayo Foundation, Rochester, Minn.

Although cocaine hydrochloride is a very useful drug, it is also very toxic. The frequency of untoward reactions has been decreased by premedication with one of the barbiturate series of drugs but recent reports[1,2] indicate that fatalities still occur following the use of cocaine. Several clinicians[3–6] have found that no serious reactions occurred when they used an absolutely fresh solution of the drug and this has been our clinical experience.

Regnier, David and Joriot[7] have reported that solutions of cocaine hydrochloride that were 6 months to several years old were more toxic to roots of white lupine seedlings than were fresh solutions but we have been unable to find a report of any investigation on animals of the relation between the age and the toxicity of solutions of cocaine hydrochloride. Consequently the following experiments were done.

Method and Results. Using sterile precautions, 1% solutions of cocaine hydrochloride in sterile distilled water were made and placed in stoppered bottles of clear glass. The solutions were kept at room temperature (75° to 95°F) exposed to daylight but not to the direct rays of the sun.

Healthy adult rabbits of both sexes were used; their weights varied from 1.5 to 4.0 kg. It was determined that the intravenous injection in 10 seconds of 10 mg of cocaine hydrochloride per kilo of body weight, a 1% solution being used, was always followed by a generalized convulsion and fairly frequently by death. This dosage and technic of injection were followed throughout this experiment.

The fresh solution of cocaine hydrochloride was used during the first hour after it was prepared. The toxicity of this solution was compared with that of solutions one week, one month and 3½ months old. It seemed wise to compare the lethal effects, as the accurate

1 Derbes, V. J., and Englehardt, H. T., *J. Lab. and Clin. Med.*, 1944, **29**, 478.

2 Shumaker, H. B., Jr., *Sur.*, 1941, **10**, 134.

3 Gardner, J. A., *J. Urol.*, 1923, **10**, 509.

4 Braasch, W. F., quoted by Hirschfelder, A. D., and Bieter, R. N., *Physiol. Rev.*, 1932, **12**, 190.

5 Emmett, J. L., *Local Anesthesia of the Urethra and Bladder;* in Lundy, J. S., *Clinical Anesthesia*, Philadelphia, W. B. Saunders Co., 1942, 195.

6 Moersch, H. J., personal communication.

7 Regnier, Jean, David, Robert, and Joriot, Robert, *Comp. rend Soc. de biol.*, 1937, **125**, 1012.

TABLE I.
Toxicity of Solutions of Cocaine Hydrochloride.*

Age of solution	Rabbits	Deaths	Mortality rate %
Fresh	76	15	19.7
1 wk	87	25	28.7
1 mo	36	9	25.0
3½ ''	10	1	10.0

* The drug was injected intravenously in 10 seconds in the dosage of 10 mg per kilo of body weight, a 1% solution being used.

grading of convulsions seemed impossible (Table I). It will be seen that there was not a demonstrable difference in the toxicity of the solutions of various ages. There was no evidence that the sex or the weight of the rabbits had any influence on the mortality rate. Neither was there any evidence that the fresh solution of cocaine hydrochloride became more toxic during the hour-long period that it was being used.

These results do not support the clinical impression that fresh solutions of cocaine hydrochloride are less toxic than older ones. However, it has been shown that sensitivity to cocaine hydrochloride varies among members of the animal kingdom, the monkey being much more sensitive than the rabbit.[8] It seems probable that the human being is even more sensitive. If this is true, then it seems possible that small differences in toxicity which could not be detected in the rabbit might be of real significance in human beings. Therefore, even though the older solutions of cocaine hydrochloride did not cause a higher death rate in rabbits, it should not be concluded that old solutions of cocaine are entirely safe for human beings. In view of the clinical reports cited in the earlier part of this paper, it would seem wise to use fresh solutions of cocaine exclusively in clinical work.

Summary. The toxicity of solutions of cocaine hydrochloride of various ages (less than one hour, one week, one month and 3½ months) was studied in rabbits, the drug being given intravenously in the dosage of 10 mg per kilo of body weight. The mortality rates following injections of solutions of cocaine of various ages showed no significant differences.

[8] Tatum, A. L., and Collins, A. H., *Arch. Int. Med.*, 1926, **38**, 405.

15349

Protection of Dry Bacteria by Fat Against Cationic Detergents.

OTTO RAHN.

From the Laboratory of Bacteriology, N. Y. State College of Agriculture, Cornell University, Ithaca, N. Y.

Klarmann and Wright[1] have shown that quaternary ammonium compounds cause an agglutination of bacteria and precipitate them against the walls of the test tube. This causes a very coarse and irregular distribution and explains the irregular results obtained by every investigator trying to evaluate the germicidal efficiency of these compounds.

It is not clear, however, why the bacteria in these clumps die more slowly than those which remain singly in suspension. In order to prove whether these detergents are strongly adsorbed on the surfaces of bacteria, 1 g of baker's yeast was suspended in 100 ml of a 100 ppm solution of Zephiran (dimethylbenzyl alkyl ammonium chloride, also known as Roccal), the yeast removed by centrifugation, and the surface tension of the clear solution measured by the drop number method (Table I.) Nearly 20 ppm of the Zephiran had been adsorbed on the cells. This amount indicates a multimolecular adsorption layer. Bentonite particles in 1000 ppm Zephiran

[1] Klarmann, E. G., and Wright, E. S., *Soap and San. Chem.*, 1946, **22**, 125.

TABLE I.
Adsorption of Zephiran on Various Surfaces.

Solution	Surface tension
Water	72.8 dynes
50 ppm Zephiran	66.5
80 " "	62.5
100 " "	59.2
Same + 1.3% filter paper	62.2
Same + 1% bakers yeast	63.4
1000 ppm Zephiran	37
Same + 5% bentonite	73

solution increased noticeably in volume, suggesting thick adsorption layers. Such layers may retard the diffusion of dissolved molecules to the center of a clump of bacteria.

However, another fact must also be included into any explanation, namely the observation by Miller, Abrams, Huber, and Klein[2] that hands dipped in 1% Zephiran solution remain apparently sterile for about 2 hours through an imperceptible film, while the bacteria of the skin, under this film, are still alive when the film is disrupted several hours later.

This observation can be explained by assuming that the adsorbed Zephiran molecules or ions are oriented, the inactive, harmless organic end directed towards the skin and the germicidal NOH group towards the outside. The organophilic skin attracts the long organic chains, and thus, the adsorption film has one harmless side and one toxic side.

To test this explanation, experiments were made with 2 different surfaces, namely fat which is organophilic, and 25% gelatin which is hydrophilic. About 1 to 2 g butterfat of high melting point were pipetted into test-tubes, and after solidification, 0.1 ml of a Staphylococcus culture was put upon the fat surface. When the culture was perfectly dry (in a vacuum), 2 to 5 ml of a 1% Zephiran solution were poured on the dry bacteria. After various exposure times, the disinfectant was poured off, the entire tube was washed once with 50 ppm Duponol (a good antidote to Zephiran), then twice with 10 ppm Duponol, and finally, broth containing 5 ppm Duponol was poured into the tube. The fat surface was then scraped with a bent platinum wire to remove as many bacteria as possible, the tube was shaken violently, and 1 ml and 0.01 ml of the broth was plated. The counts thus obtained are extremely inaccurate because bacteria may not all be removed, or may become imbedded in the fat. The chance of contamination by the repeated washing is increased. The recovery of bacteria dried on fat and treated with water gave between 200 and 20,000 colonies. If only very few colonies are found, and not consistently, it suggests a chance contamination while larger numbers are good proof of survival.

[2] Miller, B. F., Abrams, B., Huber, D. A., and Klein, M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 174.

TABLE II.
Survivors of *Staphylococcus aureus* Exposed to Zephiran Solution in Presence of Various Surfaces.

Surface	Butterfat		
Disinfectant	1% Zephiran		
Bacteria	Dried I	Dried II	Moist II
0.5 hr	12,100	—	—
1	4,000	170	—
1.5	50,000	—	—
4	—	190	0
20	—	30	0

Surface	25% gelatin				
Disinfectant	1% Zephiran	0.1% Zephiran		0.01% Zephiran	
Bacteria	dry	dry	moist	dry	moist
0.5 hr	0	0	0	1:590	9,700
1	0	0	—	0	—
1.5	0	0	0	900	0
4	0	0	—	0	—

TABLE III.
Survivors of *Staphylococcus aureus* Exposed to Disinfectants in Presence of Various Surfaces.

Surface	Vaspar		Fat		Glass	
Bacteria	dried	moist	dried	moist	dried	moist
	Disinfectant: 8% formaldehyde					
0.5 hr	0	0	261	—	0	4
1	0	0	14	0	0	1
2	900	1	0	—	—	0
	Disinfectant: 1% ceepryn					
0.5	4,400	4	—	0	0	1
1	3,500	0	24,700	42	0	0
2	—	23	—	—	—	—
3.5	24	—	0	—	0	0
7	7	—	22	—	0	—
22	0	—	0	—	—	—

The procedure with gelatin was similar, except that finally, the gelatin was melted in the broth at 40°, and plated directly. The result is shown in Table II. Bacteria dried on fat survived the powerful disinfectant for 20 hours, while they died within half an hour when not dried. By the standard technic, 50 ppm kill *Staphylococcus aureus* in 10 minutes. Bacteria dried on gelatin did not survive half an hour. Evidently the 2 surfaces affected the bacteria differently.

Table III gives the results of a parallel experiment with Ceepryn (cetyl pyridinium chloride), and with formaldehyde. Fat exerted a definite protection against formaldehyde, but not as pronounced as against Ceepryn. Vaspar (vaseline-paraffine mixture) as a pure hydrocarbon is even more organophilic than fat. The glass surface offered no protection. Bacteria dried on sand were also killed as easily as without sand.

Mudd and Mudd[3] have shown that bacteria are drawn to the interface between oil and water and usually remain there. Only the acid-fast bacteria are drawn into the oil phase. However, Jensen[4] states: "If sodium oleate in 0.05% solution is added to the water, every species and kind of microbes pass into the oil." A Zephiran solution will probably exert an influence similar to that of sodium oleate. Although the fat was solid, it is possible that a very thin film of fat covered some of the dry bacteria. Or perhaps, the surface of bacteria is so changed by drying that it becomes organophilic and becomes saturated with fat if that is present. Such cells would be protected against quaternary ammonium compounds because they would adsorb the harmless end of the molecule.

Summary. The adsorption of quaternary ammonium compounds on cell surfaces has been proved experimentally.

The formation, on hands, of a film of cationic detergent of which only the outside is germicidal, has been explained as due to an oriented adsorption of the detergents on an organophilic surface, such as fat or paraffin.

[3] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, **40**, 659.

[4] Jensen, L. B., *Microbiology of Meats*, Garrard Press, 1945, 286.

15350 P

Alloxan-Induced Azotemia in the Rat.*

LESLIE L. BENNETT AND THURID BEHRENS.

From the Divisions of Physiology (Berkeley), Medicine (San Francisco), and Institute of Experimental Biology, University of California.

The production of renal injury by alloxan is well known and was pointed out by Dunn and McLetchie[1] in their original article on the production of alloxan diabetes in the rat. In the dog, the islet tissue is more susceptible to injury than is the kidney and thus smaller doses of alloxan are nearly selectively diabetogenic in this species. With larger doses, as Goldner and Gomori[2] have shown, sufficient renal damage is produced to cause a diabetic uremic syndrome. These experiments were undertaken to determine whether this differential sensitivity was present in the rat, and whether a dose level of alloxan could be found that would produce hyperglycemia without azotemia.

All animals used were male rats of the Long-Evans strain between 45 and 50 days of age at the time of the initial injection, and were allowed free access to the stock diet at all times. Alloxan monohydrate in aqueous solution was used and was injected intraperitoneally on a body weight basis. Animals were sacrificed 72 hours after the initial injection except in those cases where the effects of persistent diabetes were studied, in which case they were sacrificed approximately one month after the initial injection. Blood was obtained from the inferior vena cava and following deproteinization, glucose was determined by the Somogyi[3] micro-method and non-protein nitrogen was determined by the micro-Kjeldahl procedure.

In Table I are presented the data on the blood glucose and blood NPN obtained 72 hours after the initial injection of various amounts of alloxan. It will be seen that with a dosage of 200 mg per kg there was a slight rise in the mean glycemia and the azotemia although all animals did not respond. The degree of elevation of the NPN was somewhat greater than that of the blood glucose, one animal showing a blood NPN of 153 mg %, as contrasted with 34 mg %, the maximum of the control group. At a dose level of 250 mg per kg the mean blood glucose was approximately doubled, being 219 mg %; but the mean NPN was increased approximately four-fold being 115 mg %. With larger doses whether given as a single injection or as repeated smaller injections, both the hyperglycemia and azotemia were more marked. In general the severity of the histopathological changes in the kidney paralleled the degree of nitrogen retention.

That this effect on the nitrogenous constituents of the blood was due to a primary renal injury and was not due to functional renal failure secondary to the severe diabetic state was shown by the fact that the simultaneous administration of insulin prevented the extreme hyperglycemia but did not alter the azotemia.

In another series of animals which received 400 mg of alloxan per kg it was determined that 3 major components of the NPN: namely, urea N, uric acid N, and creatinine N were all elevated. The mean control urea N level was 22 mg % as compared with a mean experimental level of 165 mg %. For uric acid and creatinine the control values were 3.2 mg % and 1.7 mg % respectively as compared with 12.5 mg % and 6.7 mg % for the alloxan-treated group.

* Aided by grants from the Research Board of the University of California, and the James Foundation Grant of the Medical School.

1 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

2 Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

3 Somogyi, M., *J. Biol. Chem.*, 1937, **117**, 771.

TABLE I.
Blood Glucose and Non-protein Nitrogen Level in the Rat 72 Hours After the Initial Alloxan Administration.

Alloxan dose per kg	Blood glucose in mg %		Blood NPN in mg %	
	Mean	Range	Mean	Range
Uninj. control	129 (13)*	113-146	27 (8)	23-34
150 mg	118 (13)	85-155	32 (6)	25-39
200 "	151 (11)	108-333	66 (6)	26-153
250 "	219 (10)	123-388	115 (4)	27-215
300 "	381 (6)	156-648	201 (4)	168-235
150 " 2 consecutive days. Total dose 300 mg	354 (13)	129-668	115 (6)	61-233
200 mg 2 consecutive days. Total 400 mg	736 (8)	149†-1030	140 (8)	41-238
150 mg 3 consecutive days. Total 450 mg	549 (8)	189-955	185 (8)	95-347
200 mg 2 consecutive days plus 2 units protamine insulin daily	140 (8)	41-238	177 (5)	83-245

* The figure in parentheses indicates the number of observations in the group.
† Only one value below 600 mg %.

TABLE II.
Blood Glucose and Non-protein Nitrogen Level in Rats with Alloxan Induced Diabetes of One Month's Duration.

	Blood glucose in mg %		Blood NPN in mg %	
	Mean	Range	Mean	Range
Uninjected controls (7)*	122	76-143	27	22-30
Persistent diabetics (8)	431	381-510	41	24-70
Recovered diabetics (5)	132	111-153	30	27-31

* The figure in parentheses indicates the number of observations in the group.

In Table II are presented the data obtained from animals 31-34 days following the administration of 400 mg of alloxan per kg. It will be seen that even in those animals in which there was a persistent diabetes, there was only slight residual azotemia. The mean blood NPN in this group was 41 mg % as compared with 27 mg % for the controls or as compared with 140 mg % for animals 72 hours after the injection of the same amount of alloxan. This would seem to indicate a high degree of functional recovery of the kidney. In those animals in which diabetes was not persistent there was a complete functional recovery of the kidney insofar as the NPN was completely normal.

Summary. In rats of the Long-Evans strain there is no difference between the diabetogenic and nephrotoxic dose of alloxan as judged by hyperglycemia and azotemia. Even when diabetes persists there is nearly complete functional recovery of the kidney.

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Failure of Methionine to Reduce Nitrogen Loss in Postoperative Herniorrhaphy Patients on Restricted Diet.*

CECILIA RIEGEL, C. E. KOOP, AND R. P. GRIGGER. (Introduced by I. S. Ravdin).

From the Harrison Department of Surgical Research, and the Surgical Service, Hospital of the University of Pennsylvania, Philadelphia.

Evidence that the addition of methionine to a standard diet decreased the urinary nitrogen output of dogs was reported by Miller.[1] It seemed of interest to see whether a similar effect could be obtained in patients, particularly surgical patients during the period of reduced food intake which often follows after operation.

For reasons of availability and relative uniformity 14 male patients were selected who were undergoing unilateral herniorrhaphy. They were divided into 3 groups as follows:

1. Four controls were studied for 10 days beginning on the first postoperative day. They received no added methionine.

2. Five patients were studied for 10 days beginning on the first postoperative day. They received 6.0 g of methionine daily for the first 5 days of the study.

3. Another 5 patients were studied for 10 days beginning on the first postoperative day. They received 6.0 g of methionine daily for the second 5 days of the study.

All patients were fed daily a diet containing approximately 1,000 calories and consisting of 40.0 g of protein, 40.0 g of fat, and 130.0 g of carbohydrate. The patients were kept in bed throughout the study.

Urine was collected in 24-hour periods and the volume measured. Aliquots of the first 5 days were pooled, and aliquots of the second 5 days were pooled. Analyses were run on a portion of each pooled specimen for total nitrogen by a semi-micro Kjeldahl method. Total urinary nitrogen excretion was calculated from this. Fecal nitrogen output was not determined in this group of patients but averaged 1.0 g in 18 other patients studied.[2] In this study we used an average figure of 1.0 g daily.

Results. Table I shows average intake and average excretion of nitrogen in the individual patients in the 3 groups for the 2 5-day periods.

Plasma protein concentrations were not significantly altered during the 10-day period.

Discussion. As no significant difference was found in the controls between the mean difference in intake and output of nitrogen for the first 5 days and the mean difference in intake and output of nitrogen for the second 5 days when no methionine was given, any significant differences found in those patients receiving methionine could properly be attributed to the effect of methionine. However, although small differences were found in the average output between the "methionine-fed" period and the "no methionine" period, when analyzed statistically the differences were found not to be significant (Table I). The amount of methionine fed these patients (approximately 0.1 g per kilo of body weight) was in the same range as the dogs studied by Miller.[1] The patients were on a low total caloric diet and a low, but not starvation, intake of nitrogen. It was found by Miller, whose dogs presumably received sufficient calories for maintenance, that the effect of methionine was greater in his animals before their protein stores had become depleted. These patients prior to operation were on a normal diet, and it must

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

1 Miller, L. L., *J. Biol. Chem.*, 1944, **152**, 603.

2 Riegel, Cecilia, Koop, C. E., Drew, J., Stevens, L. W., and Rhoads, J. E., to be published.

TABLE I.
Effect of Administration of Methionine on Urinary Nitrogen Excretion.

	First 5 days			Second 5 days		
	Intake g	Urine, N. g	Diff. g	Intake g	Urine, N. g	Diff. g
			No methionine			
1	32.8	62.2	—29.4	33.6	50.9	—17.3
2	35.3	61.8	—26.5	32.0	47.1	—15.1
3	35.3	49.3	—14.0	32.4	44.7	—12.3
4	33.5	57.6	—24.1	33.1	57.1	—24.0
	Mean diff.		—23.5	Mean diff.		—17.1
			Methionine first 5 days—6.0 g daily			
5	35.9	36.3	—0.4	32.8	31.1	+1.7
6	35.0	69.1	—34.1	32.8	72.2	—39.4
7	35.1	68.1	—33.0	32.1	54.7	—22.6
8	32.2	63.0	—30.8	33.9	79.2	—45.3
9	35.7	71.6	—35.9	31.2	60.7	—29.5
	Mean diff.		—26.8	Mean diff.		—27.7
			Methionine second 5 days—6.0 g daily			
10	32.4	63.4	—31.0	36.0	51.7	—15.7
11	33.0	38.8	—5.8	35.5	44.1	—8.6
12	31.9	57.9	—26.0	35.4	62.7	—27.3
13	32.9	46.1	—13.2	33.9	44.1	—10.2
14	30.1	39.5	—9.4	32.9	46.6	—13.7
	Mean diff.		—17.1	Mean diff.		—15.1

Controls—First 5 days — mean difference 23.5 g
Second 5 " — " " 17.1 g

Diff. between means = 6.4 g S.E. diff. = 4.1

Methionine Exper.—Period with methionine M.D. 21.0 g
" without " M.D. 22.4 g

Diff. between means = 1.4 g S.E. diff. = 5.9

be presumed that their protein stores were not depleted.

Conclusion. Under the conditions of this experiment the administration of 6.0 g of methionine daily to men receiving a low caloric diet failed to show a significant protein-sparing action as indicated by the effect on urinary nitrogen excretion.

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Effectiveness of Streptomycin in Arthritis of Rats.

H. M. POWELL, W. A. JAMIESON, AND R. M. RICE.
From the Lilly Research Laboratories, Indianapolis, Ind.

Two years ago we reported that penicillin was ineffective in the chemotherapy of arthritic rats infected with pleuropneumonia-like organisms, while myochrysine was effective in this respect, but at the same time quite toxic and hazardous to use in these animals.[1] Electron morphology of the micro-organisms we used was dealt with at about

[1] Powell, H. M., and Rice, R. M., *J. Lab. and Clin. Med.*, 1944, **29**, 372.

the same time in a report by Weiss[2] from this laboratory. Recently Dienes[3] has reviewed the morphology and nature of the entire pleuropneumonia group of organisms including rat strains such as we have used.

During the last 2 years we have tried out a rather long list of drugs, etc., in experimental chemotherapy in this field with no positive results until we recently tried streptomycin with what seems to be considerable success. The purpose of this report is to present these results showing effectiveness of streptomycin in this type of arthritis of rats.

In a routine screening test of various drugs, 0.05 cc of the same pleuropneumonia culture as used previously, and grown as described,[1] was injected intravenously into a group of white rats each of about 100 g weight. Four of these rats were treated hypodermically with streptomycin, while 6 were left as controls. Streptomycin therapy was started about an hour following infection, and 3 doses were given the first, second, and third days, or a total of 9 doses. Each dose comprised 1000 units. In this test, the 4 treated rats remained entirely free of symptoms. Five of the 6 controls developed pleuropneumonia infection, and one remained free of perceptible symptoms. Two of the 5 controls which developed infection had an overwhelming disease and were dead in 5 days, before very definite gross arthritis could appear. The other 3 controls developed disabling arthritis. All surviving animals were observed for 3 weeks. This kind of chemotherapeutic showing had not been seen previously in any pleuropneumonia-infected rats except those treated earlier with myochrysine, and the drug in this case was very toxic. By contrast the 4 streptomycin-treated rats in the present experiment appeared bright and alert, and entirely normal for 3 weeks after infection.

This initial experiment was repeated using 20 rats injected with pleuropneumonia culture as before. Ten of these were treated hypodermically with streptomycin and 10 were left as controls. Three doses of streptomycin were given on the first, second, third, and fourth days, or a total of 12 doses. Each dose comprised 3000 units. In this test 8 of the 10 treated rats remained entirely free of symptoms, while the remaining 2 showed a questionable trace of swelling in one toe of each animal for 2 days only. All 10 treated rats were bright and alert throughout the test and exhibited no evidence of drug toxicity. All 10 of the control rats, however, showed early and pronounced symptoms of pleuropneumonia infection. Three of these died in 5 days of overwhelming disease before visible arthritis usually appears. The other 7 developed disabling polyarthritis and died before the 3-weeks observation period was over. It is believed that the good showing of streptomycin in this experiment has not been equalled previously with myochrysine in our tests.

A third experiment is in progress using 33 rats injected with pleuropneumonia culture as before. Sixteen of these were treated with streptomycin and 17 were left as controls. None of the 16 treated rats show any symptoms, while all of the controls show evidence of infection (rapid respiration, loss of weight, and disinclination to move about), and beginning arthritis is apparent in these.

Further details concerning the effectiveness of streptomycin in rat arthritis are under study.

Our thanks are due Miss Dorothy McKay for cooperation and interest in the experimental chemotherapy set forth in this report.

Summary. Streptomycin appears chemotherapeutically effective against pleuropneumonia infections and resultant polyarthritis in rats, and better in this respect than certain gold salts. The streptomycin which we used contained 250 units per mg, and was supplied by Dr. J. A. Leighty.

2 Weiss, L. J., *J. Bact.*, 1944, **47**, 523.

3 Dienes, L., *J. Bact.*, 1945, **50**, 441.

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Presence of a Growth Inhibiting Substance in Raw Soybeans.

A. A. Klose, Barbara Hill, and H. L. Fevold.

From the Western Regional Research Laboratory, Albany, California, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

The low nutritional value of raw soybean proteins and the marked improvement on heating have been recognized for some time;[1,2,3] however, the fundamental mechanism by which this change takes place has not been satisfactorily explained. The experimentally demonstrated effect of heating is to make the methionine of the protein nutritionally available. Johnson, Parsons, and Steenbock[4] have shown that this effect is not brought about by increased absorption of the sulfur-containing amino acids but that heating apparently increases the utilization of these amino acids after absorption. Ham and Sandstedt[5] and Bowman[6] have demonstrated the presence of a trypsin inhibitor in the pH 4.2-soluble fraction of unheated soybean flakes, and Ham and Sandstedt suggested its possible significance in relation to the poor biological value of the raw soybean protein. They were not able at that time to demonstrate any growth-inhibiting effect of the fraction containing the trypsin inhibitor.

Recently, while this paper was in the process of review, a report by Ham, Sandstedt, and Mussehl[7] appeared which extended their observations and established a growth-retarding effect on chicks of the pH 4.2-soluble, acetone-insoluble fraction of soybeans. We had obtained similar results with rats, and these results are presented in this report.

In connection with work in progress at the Northern Regional Research Laboratory, dealing with the effects of processing on the food value of soybean proteins, we have been investigating the effects of heating on the growth-supporting properties of the separated acid-soluble (pH 4.2) and acid-insoluble proteins. In this investigation it was found that the crude pH 4.2-insoluble protein still responded to heat treatment by increased biological value. However, when the unheated acid-soluble fraction was added back to the heated acid-insoluble protein, the growth-promoting property was again reduced to that of unheated soybean proteins. It is obvious, therefore, that a growth-inhibiting substance is present in unheated soybean proteins which explains partially at least the nutritional inadequacy of raw soybean proteins.

Litters of albino rats (Sprague Dawley strain) were maintained on a stock diet of Purina Dog Chow Checkers supplemented with cod-liver oil and liver until they were approximately 40 days of age and 90 g per rat in weight. Each litter was then divided equally as far as possible among the experimental groups of 10 rats each and fed the corresponding diets and water *ad libitum*.

The soybean protein fractions were prepared in the following manner: ground hexane-extracted soybean flakes (No. 113) were stirred in a large volume (10 cc per g of flakes) of distilled water, and the mixture was adjusted to a pH of 6.7 with dilute sodium hydroxide. The resulting extract was pressed out in a small cider press and then

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1 Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, **32**, 91.

2 Hayward, J. W., Steenbock, H., and Bohstedt, G., *J. Nutrition*, 1936, **11**, 219; **12**, 275.

3 Hayward, J. W., Halpin, J. G., Holmes, C. E., Bohstedt, G., and Hart, E. B., *Poultry Science*, 1937, **16**, 3.

4 Johnson, L. M., Parsons, H. T., and Steenbock, H., *J. Nutrition*, 1939, **18**, 423.

5 Ham, W. E., and Sandstedt, R. M., *J. Biol. Chem.*, 1944, **154**, 505.

6 Bowman, D. E., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 139.

7 Ham, W. E., Sandstedt, R. M., and Mussehl, F. E., *J. Biol. Chem.*, 1945, **161**, 635.

TABLE I.

Effect of Various Soybean Protein Supplements on Average Gain in Weight of Young Rats. (5 Males + 5 Females per Group).

Diet No.	Protein supplement equivalent to 14.6% crude protein	Experiment No. I 35-day test: Avg gain g/rat/day	I 35-day test: Feed-consumption, g/rat/day	I 35-day test: Gain per g protein eaten, g	Experiment No. II 28-day test: Avg gain g/rat/day	II 28-day test: Feed-consumption, g/rat/day	II 28-day test: Gain per g protein eaten, g	Experiment No. III 18-day test: Avg gain g/rat/day	III 18-day test: Feed-consumption, g/rat/day	III 18-day test: Gain per g protein eaten, g
1	Hexane-extracted soybean flakes No. 113	1.06	10.8	0.65	1.19	11.7	0.67			
2	No. 113 steamed	2.20	12.0	1.21	2.80	12.7	1.45			
3	pH 4.2 insoluble soybean protein No. 113A	0.82	9.8	0.55	1.27	11.4	0.73			
4	No. 113A steamed	2.05	12.0	1.13	1.82	11.8	1.02	2.41	12.3	1.29
5	No. 113A " 90% pH 4.2 soluble soybean protein— No. 113B 10%	1.12*	10.8	0.68	0.84	10.7	0.52	1.16	10.6	0.72
6	No. 113A steamed 95 No. 113B 5							1.70	11.7	0.96
7	No. 113A " 90 No. 113B " 10	2.06*	11.3	1.20	2.24	11.5	1.28			
8	No. 113A " 90 No. 113A 10							2.71	12.5	1.42
9	Casein, commercial†	2.93	12.9	1.49	3.22	13.2	1.60	3.57	11.5	2.04
10	Casein + No. 113B (1.5% crude protein)†							2.56	10.4	1.45
11	Casein + No. 113B steamed (1.5% crude protein)†							4.19	11.6	2.12

* Diet 7 was fed for 5 days to the group of rats which received Diet 5 for the first 30 days of the test.

† The data for 9, 10, and 11 (Exp. 3) are comparable, but not comparable with the rest of the data. Younger rats were used in these 3 groups.

centrifuged in a Sharples centrifuge to remove suspended insoluble material. A second extraction of the press cake was made in a similar fashion. The combined extracts were adjusted to pH 4.2 with dilute (1*N*) hydrochloric acid. In Exp. I the pH 4.2-insoluble material (No. 113A) was centrifuged relatively free of filtrate and then dried *in vacuo* from the frozen state. In Exp. II the pH 4.2-insoluble material was redissolved by addition of alkali and reprecipitated at pH 4.2 4 times, after which it was also dried by lyophilization. Sufficient ammonium sulfate was added to the combined supernatant filtrates from the centrifugation to bring the concentration to 2.7 *M*. The resulting precipitate (No. 113B) was separated by centrifugation, dialyzed in Visking casings against distilled water at 1°C until free of ammonium sulfate, and then dried *in vacuo* from the frozen state. An optimum heat treatment of the soybean protein was accomplished by steaming in an autoclave for 30 minutes at atmospheric pressure.

All diets were composed of 50% basal mixture, a percentage of protein supplement equivalent to 14.6% crude protein, and enough of a 50-50 mixture of starch and sucrose to make 100%. Crude protein percentage was calculated by using 6.38 $\times$ % *N* for casein and 5.71 $\times$ % *N* for the soybean proteins. The basal mixture, making up 50% of each diet, contained (as percent of the complete diet) cottonseed (Wesson) oil 5, U. S. P. cod-liver oil 2,34% tocopherol vegetable oil concentrate 0.05, salt mixture (McCollum's No. 185 + trace elements) 4, corn starch 18.5, sucrose 18.5, brewers' yeast 2, and (as mg per 100 g of the complete diet) choline chloride 50, thiamin chloride 0.2, riboflavin 0.5, pyridoxin 0.2, calcium pantothenate 2.5, and nicotinic acid 1.0.

Table I presents the results obtained on feeding the acid-soluble (pH 4.2) and acid-insoluble proteins in various combinations. The marked growth-retarding effect of the unheated, pH 4.2-soluble, 2.7 *M* ammonium sulfate-insoluble fraction when allowed to replace 10% of the heated pH 4.2-insoluble fraction and the dissipation of the effect on heating, is at once apparent. It is also obvious that the growth inhibitor is concentrated in the 10% of pH 4.2-soluble protein, since the effect of the inhibitor contained in 10% of soluble protein, when introduced in the diet, is greater than that of the inhibitor contained in 10 times the amount of pH 4.2-insoluble protein (compare 3, 4, and 5, Exp. II); also, the inclusion of 10% of unheated pH 4.2-insoluble protein results in no inhibition as compared to that produced by 10% of unheated pH 4.2-soluble protein (compare 4 and 8, Exp. III). The effect of the growth inhibitor is also demonstrated when included in a diet in which casein was the protein component (9, 10, and 11, Exp. III).

The question of the identity or non-identity of the trypsin inhibitor with the growth inhibitor cannot be answered from the present data. The trypsin inhibitor is present in the acid-soluble fraction of soybean proteins, and the 2 may therefore be identical. Since the tryptic inhibitor has recently been isolated in crystalline form by Kunitz,[8] the question can best be answered by inclusion of the isolated substance in the diet. Such studies, together with further characterization of the growth-inhibiting substance, are under way and will be reported at a later date.

Summary. A substance which inhibits the growth-promoting properties of proteins in rats is present in raw soybeans. The substance is non-dialyzable, precipitated by salt, and inactivated by heat. It appears, therefore, to be a protein, which has been shown to be concentrated in the acid-soluble (pH 4.2) fraction of the soybean protein. These results are in agreement with those presented by Ham, Sandstedt, and Mussehl[7] for the chick.

[8] Kunitz, M., *Science*, 1945, **101**, 668.

15354

Urinary Output and Phosphorus Excretion in Human Subjects During Prolonged Exposures at Low Simulated Altitudes.*

SAVINO A. D'ANGELO. (Introduced by Harry A. Charipper).

From the Aero Medical Laboratory,† Wright Field, Dayton, Ohio, and the Department of Biology, Washington Square College of Arts and Sciences, New York University.‡

Among the many described effects of anoxia on the organism, those on the urinary secretion and the mineral metabolism are especially difficult of interpretation. Van Liere[1] has reviewed the evidence bearing on the problem and has indicated its controversial status. In neither the lower mammal nor in man are the results in good agreement. The anesthetized dog generally displays oliguria while breathing at reduced oxygen tensions equivalent to altitudes of 14,000 ft. and above, yet considerable variation and polyuria may occur.[2,3,4] In the unanesthetized animal at 18,000-20,000 ft. simulated altitudes polyuria appears to be the typical response.[5,6] Reports on the urinary output of the anoxic human being are also discordant. McFarland and Edwards[7] described increased urine elimination in certain of the crew, but not the passengers, at the beginning of prolonged trans-Pacific flights at altitudes of 8,000 to 12,000 ft. The diuresis was marked in the chronic anoxia experiments of Armstrong and Heim.[8] Urine output was increased from 100 to 300% of normal in subjects maintained for 4 or 7 hours daily at a 12,000 ft. simulated altitude. Bryan and Ricketts,[9] on the other hand, obtained essentially negative results at comparable as well as higher altitudes (11,500-18,000 ft.)

The scanty reports regarding the effect of altitude on the mineral metabolism are similarly conflicting. Acute anoxia in the dog produces a pronounced increase in the renal excretion of sodium, potassium, chloride, as well as lesser increases in the excretion of nitrogen and phosphorus.[5,6] These changes do not occur in the human subject. The species difference is particularly marked as regards phosphorus metabolism. Sundstroem,[10] Lewis *et al.*[6] and Bryan and Ricketts[9] have all demonstrated that phosphorus excretion in the human being is decreased rather than increased in anoxia. The physiological significance of this effect is still unclear. The present study represents one phase of a research project investigating the physiological responses of the human subject during prolonged exposures at moderately low simulated altitudes. The effects on the carbohydrate

* These experiments were done at the Aero Medical Laboratory of the Engineering Division, Air Technical Service Command, while the author was assigned there as an aviation physiologist.

† The author wishes to express appreciation to C. Kwoka and S. Kosky for technical assistance, and also to other enlisted personnel of the aero medical laboratory for their cooperation in this investigation.

‡ Present address.

1 Van Liere, E. J., *Anoxia, Its Effect on the Body,* Univ. of Chicago Press, Chicago, 1942.

2 Van Liere, E. J., Parker, A. S., Crisler, G. R., and Hall, J. E., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 479.

3 Toth, L. A., *Am. J. Phy.*, 1937, **119**, 127.

4 Malmejac, J., *J. Av. Med.*, 1944, **15**, 167.

5 Langley, L. L., and Clarke, R. W., *Yale J. Biol. and Med.*, 1942, **14**, 529.

6 Lewis, R. A., Thorn, G. W., Koepf, G. F., and Dorrance, S. S., *J. Clin. Invest.*, 1942, **21**, 33.

7 McFarland, R. A., and Edwards, H. T., *J. Av. Med.*, 1937, **8**, 156.

8 Armstrong, H. G., *Principles and Practices of Aviation Medicine,* Baltimore, Williams and Wilkins Co., 1939, 284.

9 Bryan, H. A., and Ricketts, H. T., *J. Clin. Endocrinol.*, 1944, **4**, 450.

10 Sundstroem, E. S., *Univ. California Publ. Physiol.*, 1919, **5**, 121.

and respiratory metabolism have already been reported.[11,12] This investigation presents data on the urinary output and the renal excretion of phosphorus in individuals maintained in the low pressure chamber at altitudes of 8,000 and 10,000 ft. for periods of 10 hours without supplementary oxygen.

Materials and Methods. The detailed procedure for the experiment has been previously described.[12] The data are based on a total of 72 man runs and represent additional measurements taken in the foregoing investigation. Each of the 5 subjects received at least 6 runs at ground level (800 ft.) and a minimum of 5 runs at the 8,000 or 10,000 ft. level. Flights were taken once weekly and alternated between altitude and ground level. Subjects reported to the laboratory in the post-absorptive state. No attempt was made to control the amount nor composition of the meal taken at the regular mess 12-15 hours the evening before. Various metabolic measurements were made in the initial fasting state and all urine was voided at this time. Each subject was then given a standard K-ration breakfast (884 cal.) with 250 cc of water. After consumption of this meal, the chamber was brought to the desired pressure altitude, and the subjects maintained there at rest for 10 hours breathing ambient air and without food. Although water was allowed *ad libitum* in the major experiment, the present data are based on runs during which no water was taken throughout exposure. The control runs were done in the pressure chamber under otherwise identical conditions. Urine collections were made at the end of the first, third, fifth, seventh, and tenth hours of exposure (the "first hour" collection period represents a 2-hour sample, that is, the time from which the initial fasting urine was voided to the end of the first hour of the experiment proper). Urinary phosphorus was determined by the method of Fiske and Subbarow.[13]

Results. Examination of the data in Table I reveals that the total output of urine at both altitudes studied was not appreciably altered from the ground-level condition. The mean hourly secretion of urine (all subjects combined), as averaged for the entire exposure period, was 55 cc at ground level as against 46 cc (2 subjects) and 59 cc at the 8,000 and 10,000 ft. altitudes respectively. In only one individual (G. B.) was the urine volume consistently and significantly increased at altitude (10,000 ft.) The mean hourly secretion in this instance was 57 cc at ground level and 76 cc at altitude, a mean increase of 25.9% at the 10,000 ft. level.

The characteristic feature of urine elimination, as seen in this study, was the considerable degree of variation found in any individual at ground level or altitude. Urine volumes at corresponding time intervals varied widely in successive runs. As little as 200 cc or as much as 1200 cc of urine were voided over the entire 11-hour period on some occasions. The time in exposure at which maximal elimination occurred also differed among subjects. The highest rates of urine output were found within the first half of the exposure period at ground level or altitude, and very likely represented the elimination of the water ingested with the standard breakfast. There was no evidence to indicate that significant differences existed between ground level and altitude as regards urinary output in early as against late exposure. Total urine elimination for the entire experimental period was of the same order of magnitude in all subjects.

The renal excretion of inorganic phosphorus was significantly decreased in all subjects at altitude (Table II). The reduction bore no apparent relationship to urine volume (Table 1). The mean total phosphorus output for the entire exposure period (all subjects combined) was 332 mg (range, 259-392 mg) at ground level, 244 mg (range, 227-261 mg) at 8,000 ft., and 233 mg (range, 195-298 mg) at the 10,000 ft. level. These values represent a mean decrease from the ground level excretion of 29.4% and 22.6% at the higher and lower altitude respectively. Figures for the 8,000 ft. level are based on determinations made in 2 subjects only, in one of

[11] D'Angelo, S. A., *Am. J. Phy.*, 1946, **145**, 365.

[12] D'Angelo, S. A., *Am. J. Phy.*, in press.

[13] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

TABLE I.
Urinary Output at Ground Level and Altitude with Exposure Time.

Subject	Time (hr)	Ground level (800 ft.) Vol. (cc)	Ground level (800 ft.) Rate‡ (cc/hr)	8,000 ft. Vol. (cc)	8,000 ft. Rate (cc/hr)	10,000 ft. Vol. (cc)	10,000 ft. Rate (cc/hr)
	*	†					
	IF-1	123±32		152±33		153±54	
R.B.	1-5	262±43		235±18		302±74	
	5-10	184±25	52	196±44	53	198±40	59
	IF-1	55±27				54±24	
S.K.	1-5	452±112				456±135	
	5-10	188±48	63			164±42	61
	IF-1	86±21		84±17		65±48	
L.K.	1-5	232±22		176±48		254±34	
	5-10	242±52	51	172±50	39	242±64	51
	IF-1	151±49				130±35	
G.B.	1-5	266±50				316±52	
	5-10	206±22	57			394±47	76
	IF-1	74±22				66±18	
C.K.	1-5	237±30				212±40	
	5-10	275±42	53			279±51	51
Combined avg			55		46		60

* Represents a 2-hour interval which includes an hour period at ground level when initial fasting determinations were made and breakfast consumed, and the first hour of the experimental period proper.

† Represents the mean urine volume ± the standard deviation.

‡ Refers to the mean hourly secretion of urine over the 11-hour period of observation.

TABLE II.
Phosphorus Excretion at Ground Level and Altitude with Exposure Time.

Subject	Time (hr)	Ground level P. excr. (mg/hr)	Ground level Total P.†	8,000 ft. P. excr. (mg/hr)	8,000 ft. Total P.	10,000 ft. P. excr. (mg/hr)	10,000 ft. Total P.	% decrease at alt. 8,000	% decrease at alt. 10,000
	IF-1	30.8(2.3)*		29.3(3.6)		25.4(4.3)			
R.B.	1-5	29.4(2.6)		20.0(3.9)		17.7(3.4)			
	5-10	38.4(2.0)	371	19.6(5.1)	227	20.8(2.3)	226	38.9	39.2
	IF-1	7.5(2.0)				10.0(0.8)			
S.K.	1-5	27.9(3.6)				20.1(2.1)			
	5-10	26.5(1.7)	259			20.6(2.3)	203		21.5
	IF-1	18.3(2.9)		28.4(2.6)		16.1(3.5)			
L.K.	1-5	20.3(2.3)		15.1(2.5)		15.5(2.0)			
	5-10	32.1(2.6)	278	28.7(5.6)	261	20.1(1.4)	195	6.3	30.0
	IF-1	25.6(4.1)				22.2(2.5)			
G.B.	1-5	32.3(5.0)				23.0(3.5)			
	5-10	42.3(3.2)	392			32.3(2.9)	298		23.9
	IF-1	15.0(2.1)				12.5(2.2)			
C.K.	1-5	23.6(4.1)				20.5(3.2)			
	5-10	47.4(3.2)	361			27.6(3.3)	245		32.2
Combined avg			332		244		233	22.6	29.4

* Represents the mean rate of phosphorus excretion, and in parentheses, the standard error of the mean.

† Refers to the total phosphorus excretion for the 11-hour period.

whom phosphorus excretion was just slightly diminished.

Excretion rates at ground level increased with exposure time so that the phosphorus content of urine was substantially greater during the last half of the experimental period. This increase with continuing exposure was either lessened or abolished at altitude, the diminution becoming apparent at either altitude level within the first half of the exposure period. Aside from relatively high values of phosphorus excretion during the first-hour interval in one subject, subsequent values at 8,000 ft. approximated those at the higher level and were significantly lower than those at ground level for corresponding time intervals.

Discussion. The results of these experiments indicate that urinary water secretion is not appreciably affected in the resting subject during prolonged exposures at moderately low altitudes. These findings are in accord with those of Bryan and Ricketts[9] at higher altitudes but fail to confirm the results of Armstrong and Heim[8] at the 12,000 ft. level. The former group exposed subjects to altitude pressures of 11,500, 16,600 and 18,000 ft., for 4 to 6 hours, 6 days a week, from 4 to 6 weeks under controlled metabolic conditions. In only one individual was there a significant increase in the volume of urine passed at altitude (16,600 ft.) McFarland and Edwards[7] did describe increased urine elimination in actual flight at altitudes comparable to those in this study. The polyuria, however, was best associated with the nervous tension of piloting the ship. Diet and fluid intake, moreover, were not controlled.

The considerable variation found in the output of urine, despite controlled conditions of food and water intake, indicates the importance in studies such as these of making sufficient measurements over reasonably long periods of observation. In this regard, it must be stated that the 24-hour urine volume was not determined, nor was the fluid intake for each subject known prior to the experimental period. It is believed, however, that determination of the urine volume over an 11-hour period should reveal any existing effect of moderately low altitude on urinary output. There was no indication that at altitude an initial polyuria occurred which was subsequently compensated for by oliguria, as may occur in the rat. Silvette[14] described a marked and sustained polyuria in rats given daily 3-hour exposures at 15,000 and 25,000 ft., whereas, Swann and colleagues[15,16] found no appreciable change in urine output, despite negative water balances, in longer exposures (6-24 hours) at an 18,000 ft. level.

It becomes evident as studies on anoxia continue that physiological changes begin to occur in the body at altitudes lower than previously thought. The present study indicates that the renal excretion of phosphorus may be affected at altitudes as low as 8,000 ft. We can advance no explanation for this effect at present. Since it is elicited at ground level breathing appropriate nitrogen-oxygen mixtures, it is more clearly attributable to the reduced oxygen tension than the reduced barometric pressure *per se*.[6] In the experiments of Bryan and Ricketts[9] the excretion of phosphorus was definitely decreased in the urine passed at altitude, but the 24-hour output was unchanged. Urinary calcium decreased also, but in a manner unrelated to phosphorus.

Whether or not the diminished excretion of phosphorus is primarily a reflection of an acid-base shift in the anoxic organism, or whether it fundamentally reflects some change in the intermediary metabolism is not clear. Alteration in phosphate concentration of the body fluids in acidotic and alkalotic states is well established. The intimate relationship between phosphorus and carbohydrate metabolism is also well known, and needs no elaboration here. In this study, the phosphorus excretion could not be correlated directly with changes in the respiratory metabolism.[12] Although phosphorus output was decreased to a greater extent in sub-

[14] Silvette, H., *Am. J. Phy.*, 1943-44, **140**, 374.

[15] Swann, H. G., Collings, W. D., Cline, J. K., and Dernehl, C. U., *Science*, 1942, **96**, 588.

[16] Collings, W. D., Swann, H. G., Dernehl, C. U., and Cline, J. K., *Fed. Proc.*, 1943, **2**, 7.

jects showing respiratory alkalosis, it appears significant that in the one subject (S. K.) displaying no change in respiration urine phosphorus was appreciably diminished. The changes in the renal excretion of inorganic phosphorus may well reflect some change in the carbohydrate metabolism. Leipert and Kellersman[17] found in human subjects at 5500-6200 meters that a decrease in the phosphorus of blood and urine was compensated for by an increase of phosphate ester in the blood, an effect further augmented by glucose. Similar changes in rats could be correlated with greatly increased liver glycogen. Whether this relationship existed in the present experiments at moderately low altitudes cannot be stated. In this connection, it was found that the blood sugar level remained unaffected at the 8,000 and 10,000 ft. levels.[11]

Conclusions. 1. No appreciable change in total urine output from ground level values was found to occur in human subjects during prolonged exposures to simulated altitudes of 8,000 and 10,000 ft. under conditions of restricted food and water intake.

2. The renal excretion of phosphorus was significantly decreased at altitude.

3. These experiments indicate that alteration in the mineral metabolism may occur in the unacclimatized human being at altitudes as low as 8,000 ft.

[17] Leipert, T., and Kellersman, E., *Z. Physiol. Chem.*, 1942, **276**, 214.

15355

Blood Agglutinins in Filariasis.

M. B. FRANKS.* (Introduced by H. M. Zimmerman).
From the United States Naval Medical Research Unit No. 2.†

In a recent study of filariasis on Okinawa,[1] data on the frequency distribution of the A, B blood groups were collected. Certain differences in the frequencies of the blood groups, in comparison to the systematic variation in the general population, were noted to occur in filarial patients who had circulating microfilariae; these differences warrant a brief note at this time.

The blood-group percentages of 1,000 persons from the villages of Fukeyama, Jinusza and Soke[2] (approximately 5% of the population) were compared with the frequency distribution of the A, B blood groups in 180 persons, from the same villages, who had circulating microfilariae. The degree of microfilaremia was determined by dilution counting[1] and its relationship to the blood group percentages was also studied. As noted in Table I, the incidence of filaremia is greater in persons whose sera contain no natural antibody to group A red blood cells. When the data were examined without reference to the variables that influence the degree of filaremia, it was found that persons whose blood did not contain α-agglutinins had, as a rule, higher microfilarial counts. High α- and β-isoagglutinin titers (800-1600) were observed in the sera of many filarial patients, even in those who did not have microfilaremia. In several instances, the sera contained ag-

* The writer is indebted to Comdr. Harold Fink, MC, USNR, who rechecked the blood group typings on all of the filarial patients, and who is responsible for the compilation of the A, B blood group percentages of the Okinawans.

† The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse the views expressed in this paper.

[1] Franks, M. B., Chenoweth, B. M., Jr., and Stoll, N. R., *Am. J. Trop. Med.*, 1946, in press.

[2] Fink, Harold, report to be submitted.

TABLE I.
Frequencies of the A, B Blood Groups in Persons with Microfilaremia Compared with the Systematic Variations in the Frequencies of the Blood Groups on Okinawa.

	A	AB	O	B
% in 1,000 Okinawans*	40.8	8.3	31.8	19.1
% in 180 persons with filaremia	52.0	10.3	25.9	11.8
†Statistical significance of differences	Significant	Not significant	Possibly significant	Significant

* Comdr. Fink has since done 500 additional typings from these villages without any appreciable change in percentages given here.

† The odds against the occurrence of the differences in the ratios arising by chance alone are approximately 40 to 1 for A, 1 to 1 for AB, 10 to 1 for O, and 140 to 1 for B.

glutinins active for the agglutinogens present in their cells. One group A and 4 group AB patients gave anomalous reactions of this type. The α-agglutinins were found in 4 of these cases and were present in low titer.

The possible physiological function of the blood group substance is not known. The serological relationship of A substance to some bacterial carbohydrates suggests that the blood group substances may have an immunological role. However, there is no experimental evidence that one blood group or type enjoys any advantage over the other in this respect.

Oliver-Gonzalez[3,4] has been able to demonstrate that the polysaccharides of several helminths (originally isolated by Campbell from *Ascaris lumbricoides*) inhibits the α- and β-agglutinins of human sera. Although a similar polysaccharide has not been isolated in the filarid, it is possible that such a substance related to the blood group substances exists and might account for the relationship noted above in the filarial patients. In another experiment, Oliver-Gonzalez[5] reported extraordinarily high isoagglutinin titers in malaria patients with blackwater fever, suggesting that the malaria parasite contains blood group substances or related antigens. Heidelberger and Mayer[6] recently noted that persons of blood group O or B, who were vaccinated with malaria parasites, were more likely to cease relapsing than persons of blood group A or AB. They also demonstrated significant rises in agglutinin titers of the blood group O or B volunteers whom they injected with malaria vaccine prepared from infected O or B blood. Control subjects injected with normal human blood group O stromata showed no changes in their α-agglutinin titers.

The data here are not detailed enough to determine the immunological significance of the presence of autoagglutinins in the 5 sera from filarial patients. An attractive hypothesis is one that provides for the production of the agglutinin as the result of the stimulation by a blood group-like substance in the filarid. However, our present knowledge lends no support to such a view. The data are difficult to assess, but the suggestion that there might be a relationship between the natural antibodies of the red blood cells and the antibodies that have a selective action on an organism, indicates the desirability of adding information along these lines.

[3] Oliver-Gonzalez, Jose, *J. Infect. Dis.*, 1944, **74**, 81.

[4] Oliver-Gonzalez, Jose, and Torregrosa, M. V., *J. Infect. Dis.*, 1944, **74**, 173.

[5] Oliver-Gonzalez, Jose, and Montilla, E., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 169.

[6] Heidelberger, Michael, and Mayer, Manfred, *N. R. C. Bulletin on Malaria Research*, September 20, 1944.

15356

Toxicity of Nicotinic Acid and Some of Its Derivatives.

Fred G. Brazda and R. A. Coulson.

From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans.

Handler and Dann[1] observed that the incorporation of large amounts of nicotinamide into the diet caused a decreased growth rate in young rats whereas nicotinic acid and trigonelline had little effect. It was concluded that the decreased growth rate was due to the depletion of the stores of methionine (which was deemed essential for the methylation of nicotinamide) since in the process of detoxication nicotinamide was methylated and excreted as what appeared to be trigonelline. Later investigations[2] indicate that the principal detoxication product is more probably nicotinamide methochloride (N^1-methylnicotinamide).

The marked toxicity of nicotinamide when administered subcutaneously leads one to suspect that the molecule itself exerts a toxic action which is independent of the effect of the compound on the depletion of the stores of "methyl donors." To determine the influence of molecular configuration on toxicity highly purified preparations of pyridine, nicotinic acid (as the sodium salt), nicotinamide, coramine (N,N-diethylnicotinamide), and the respective methyl derivatives of these compounds were administered subcutaneously to young rats of both sexes weighing between 50 and 100 g. The dose needed to kill one-half of the animals in each group was determined. The results appear in Table I.

TABLE I.
Relative Toxicity of Some Derivatives of Pyridine (Subcutaneous Injection).

	LD_{50}* g/kilo	Relative-order of toxicity
Pyridine	1.00	5.00
Pyridine methochloride	0.28	17.9
Nicotinic acid	5.0	1.0
Trigonelline	5.0	1.0
Nicotinamide	1.68	3.0
Nicotinamide methochloride	2.40	2.08
Coramine	0.24	20.8
Coramine methochloride	1.90	2.63

* LD_{50} = dose needed to kill one-half of the animals.

Methylation decreases the toxicity of coramine and nicotinamide, increases the toxicity of pyridine and has little or no apparent influence on the toxicity of nicotinic acid. It is certain that methylation is not the only factor concerned in the toxicity of these compounds since nicotinamide methochloride is more toxic than nicotinic acid. If pyridine is methylated in the rat as it is in the dog[3] this is an example of the conversion of a toxic compound into one which is even more toxic in the process of "detoxication."

Nicotinamide, coramine and the methyl derivatives of these compounds cause paralysis of the respiratory center when administered in large doses. The injection of pyridine and pyridine methochloride in amounts slightly below the lethal dose produced a deep anesthesia of about 2 hours' duration. These compounds had no apparent effect on respiration. Trigonelline and nicotinic acid have such a low order of toxicity that an accurate estimation is impossible since in the massive doses employed the principal effect may be due to the administration of large amounts of hypertonic solutions.

With regard to the effect of the configura-

[1] Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **146**, 357.

[2] Huff, J. W., and Perlzweig, W. A., *Science*, 1943, **97**, 538; Ellinger, P., and Coulson, R. A., *Nature*, London, 1943, **152**, 383.

[3] His, W., *Arch. exp. Path. u. Pharm.*, 1887, **22**, 253.

tion on the β position of pyridine it is evident that the presence of the carboxyl group decreases the toxicity of the molecule. Conversion of this group to the simple amide markedly increases toxicity; the presence of a substituted amide on this position increases the toxicity so that it exceeds that of the unsubstituted pyridine. The high toxicity of coramine suggests the necessity for caution in its administration.

Summary. The relative toxicity of subcutaneous injections of nicotinic acid, nicotinamide, coramine, pyridine and their respective methyl derivatives has been determined. Methylation decreases the toxicity of coramine and nicotinamide, increases the toxicity of pyridine and has little or no apparent influence on the toxicity of nicotinic acid. The toxicity of the non-methylated compounds appears to be due directly to the structure of the compounds rather than to the depletion of the body stores of methyl donors in the process of detoxication.

15357

Distribution of 2,2 (p-Chlorophenyl) 1,1,1 Trichlorethane (DDT) in Tissues of Rats after Its Ingestion.*

STEPHAN LUDEWIG AND ALFRED CHANUTIN. (Introduced by H. E. Jordan).

From the Biochemical Laboratory, University of Virginia, Charlottesville.

The data for the distribution of DDT in tissues of experimental animals fed DDT are limited. Smith and Stohlmann[1,2] analyzed blood, kidney, liver, central nervous-system and bile after acute and chronic poisoning in rabbits and cats by determining organic chloride. The highest DDT concentration was observed in the bile. Laug[3] determined the DDT concentration in tissues of rats fed on diets containing small amounts of DDT for periods varying from 6 months to 2 years. By far, the greatest amount of DDT was found in perirenal fat. In spleen, liver and kidney, the amount of DDT stored appeared to be roughly proportionate to the amount of total ether extractable-material in these organs. Woodward[4] studied the DDT concentrations in tissues of a chronically poisoned dog, monkey, pig and turkey. The highest concentration of DDT was found in fat; the adrenals of the dog and the monkey contained appreciably larger amounts of DDT than the liver, kidney and brain.

The distribution of DDT in the brain, liver, kidney and adrenals of rats fed diets containing 0.1 and 0.2% DDT was determined at frequent intervals.

Methods. Inbred male rats of Wistar stock, 60 to 70 days old, and weighing 150-200 g were used as experimental animals. They were maintained on a stock diet until the experimental diet was fed. The diet contained 20% casein (Labco), 8% crisco, 4% inorganic salt,[5] 65% sucrose, 2% agar, vitamin supplements and 0.1 or 0.2% DDT (M.P. 108.6-109.5°). The animals were fed *ad libitum* and were sacrificed at frequent intervals. After nembutal injection, the animals were exsanguinated and the tissues immediately removed for the DDT determination.

The tissues were prepared for analysis according to Ofner's[6] recommendation and the

* This work was done under contract with the Medical Division of the Chemical Warfare Service.

1 Smith, M. I., and Stohlman, E. F., *Pub. Health Rep.*, 1944, **59**, 984.

2 Smith, M. I., and Stohlman, E. F., *Pub. Health Rep.*, 1945, **60**, 289.

3 Laug, E. P., personal communication.

4 Woodward, G., personal communication.

5 Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, **15**, 317.

6 OSRD Insect Control Committee, Report No. 65.

Fig. 1.
Distribution of DDT in brain, kidney, liver and adrenals of rats after ingesting diets containing 0.1 and 0.2% DDT.

DDT was determined by the Schechter-Haller[7] colorimetric method. Both adrenals and aliquots of kidney, liver and brain extracts were used for analyses. The Evelyn photometer with filter No. 600 was used. Standard solutions of DDT were analyzed with each set of determinations to serve as checks for accuracy of the analysis.

Results. The results of the analyses are shown in Fig. 1. The tissues of animals fed the diet containing 0.1% DDT store small amounts of the agent and the concentrations of DDT remain constant during the 5-15-day period of observation. The animals gained weight despite the characteristic tremors while on this experimental diet and no deaths occurred during the period of observation.

The DDT concentrations of the brain and kidney of rats on the diet containing 0.2% DDT increase slightly on the 7th and 11th days. It is surprising that the brain, which is rich in total lipids, stores only a small amount of DDT. The concentration in the liver appears to reach a maximum on the 4th day. There is a marked and significant increase in the DDT concentration in the adrenals which reaches its maximum on the 11th day. The adrenals are comparatively rich in lipids, but no data concerning the changes in the amount and character of these lipids are available. All animals lost weight and approximately half died during the first 9 days.

Summary. The DDT contents of the liver, kidney, brain and adrenals of rats fed diets containing 0.1% and 0.2% DDT were determined at various intervals.

The amounts of this agent stored in the respective tissues reaches a maximum within a few days and remains at a constant level in animals fed 0.1% DDT. The greatest concentration was noted in the adrenals.

The concentrations of DDT in the brain, liver and kidney of rats fed 0.2% DDT increase slightly and progressively with time. The concentration of this material is markedly increased in the adrenal at the time when the animals are severely intoxicated.

[7] Schechter, M. S., Soloway, S. B., Hayes, R. A., and Haller, H. L., *Ind. and Eng. Chem., Analyt. Ed.*, 1945, **17**, 704.

15358

Age and Species Variation in the Acute Toxicity of Alpha-Naphthyl Thiourea.*

Sally H. Dieke and Curt P. Richter.†

From the Psychobiological Laboratory, Phipps Psychiatric Clinic, The Johns Hopkins Hospital, Baltimore, Md.

The general properties of α-naphthyl thiourea (ANTU) have been described in a previous paper,[1] in which it was shown that this substance, besides being an effective poison for the field control of Norway rats, has interesting possibilities as a tool for research because of its actions leading to pulmonary edema, increased lymph flow[2] and other physiological and anatomical effects.[3,4]

The present work was performed to elucidate the practical questions of whether ANTU is toxic to other animals than Norway rats and would therefore present hazards in use, and also whether, as was indicated in field trials, young Norway rats are less susceptible than adults. Differences between males and females were sought at the same time.

Although our primary objectives were practical ones, bearing on the use of ANTU as a rodenticide, the results obtained are presented here because of the many applications of ANTU and other thiourea derivatives to various fields of research.

Age and Sex Variation in Rats—Wild Norway rats were chosen for test animals, because of the large strain differences in response to thiourea poisoning which had been found between wild and laboratory rats,[5] and which it was felt might exist for ANTU as well.

The rats were all wild brown Norways, trapped in the alleys and back yards of Baltimore, Maryland, in special wooden box traps which have been described elsewhere.[6] They were predominantly from residential districts. Care was taken to use only rats trapped in areas where no systematic field poisoning with ANTU had been done. All rats (except some classed as suckling) were held in the laboratory a minimum of 4 days after trapping to allow them to recover from that ordeal and to make sure they were healthy. During this period they were fed Purina fox chow cubes and had access to an ample water supply.

The ANTU was suspended in olive oil and administered by intraperitoneal injection, the dose being adjusted to body weight by giving a volume corresponding to 0.1 cc per 10 g body weight. The rats were not starved; they were, however, injected in the late afternoon, just before the normal feeding time of wild rats.

To restrain the rats for weighing and injecting use was made of the "sock" developed by Emlen.[7] This device made it possible to

* This work was begun under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Johns Hopkins University, and completed under a contract between the Chemical Warfare Service and the Johns Hopkins University.

† We are indebted to Dr. E. B. Astwood in Boston for sending us the rats from his colony used in comparative toxicity tests, and to Mr. H. J. Spencer of the Fish and Wildlife Service in Gainesville, Florida, for trapping and shipping to us a number of wild Alexandrine rats.

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5 Dieke, S. H., and Richter, C. P., *J. Pharm. and Exp. Therap.*, 1945, **83**, 195.

6 Richter, C. P., and Emlen, J. T., Jr., *Public Health Rep.*, 1945, **60**, 1303.

7 Emlen, J. T., Jr., *J. Wildlife Management*, 1944, **8**, 264.

TABLE I.
Age and Sex Variation in Susceptibility of Wild Norway Rats to ANTU Poisoning.

Wt. range (g)		No. rats used Male	Female	Total	Average body wt. (g)	$LD_{50} \pm$ S.E. (mg/kg body wt.)
		Age				
0- 50	suckling	17	7	24	39.3	58±4.4
51-100	weanling	23	17	40	80.7	43±5.7
101-125	young	11	24	35	112.5	22±3.2
126-150	''	15	7	22	140.4	18±4.3
151-200	''	15	14	29	170.1	16±2.7
201-300	young adult	10	26	36	263.1	8.1±0.9
301-400	adult	45	37	82	348.3	7.7±1.0
401-546	''	39	17	56	447.6	6.2±0.6
Total				324		
		Sex				
Above 301	(adult males)	84	—	—		7.0±0.7
'' 301	('' females)	—	54	—		7.4±0.9

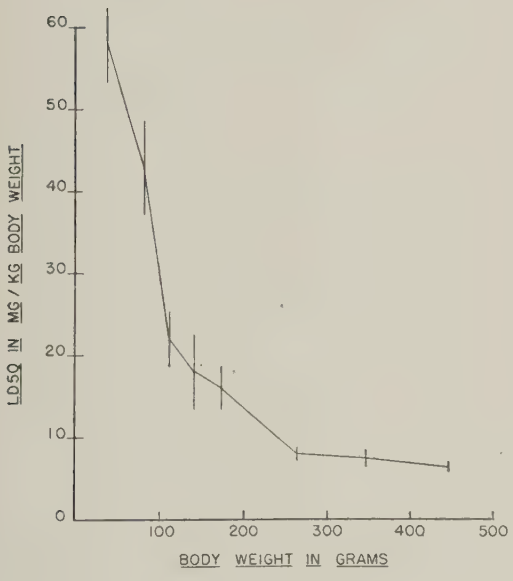

Fig. 1.

Change in acute LD_{50} of ANTU with body weight of wild Norway rats. The LD_{50} for each weight range is plotted against an abscissa representing the average body weight of rats in that range. The standard error of each LD_{50} is indicated by a vertical line of appropriate length.

handle wild rats without anaesthetics in much the same way as laboratory rats.

The assays on rats weighing more than 200 g were carried out over the course of a year and a half (January 1944 to June 1945) with no seasonal variation being noted. The assays on rats below 200 g were performed during April, May and June 1945.

The results obtained with a series of 324 rats are summarized in Table I. The median lethal doses (LD_{50}'s) given in the last column were estimated, together with their standard errors, by the method of Litchfield and Fertig,[8] using logarithmic-probit graph paper.

A comparison of the LD_{50} values given in Table I, and presented graphically in Fig. 1, shows that the resistance to acute ANTU poisoning was greatest in the youngest rats, and then decreased fairly rapidly to level off between 8 and 6 mg/kg for rats weighing 200 g or more. Suckling rats (weighing less than 50 g) and weanling rats (up to 100 g) were respectively about 7 and 5 times as resistant as old rats. Between 100 and 200 g the resistance was still more than twice as great as it was above 200 g. It may be mentioned in this connection that a corresponding (but larger) difference in response to acute poisoning with the parent compound thiourea has been observed between young and adult laboratory rats.[9,5]

The correlation of weight with age, which is indicated by the classifications into suckling (birth to approximately 30 days of age), weanling (30 to 60 days) and adult (over 90 days), is only an estimate, based on observations such as whether the rats were trapped in a group with their mother, were

[8] Litchfield, J. T., Jr., and Fertig, J. W., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 276.

[9] MacKenzie, J. B., and MacKenzie, C. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 34.

TABLE II.
Estimated Acute LD_{50} Values for Various Animals (Adult).

	Administered*	No. used	LD_{50}† mg/kg body wt.
Norway rat *(Rattus norvegicus)*			
domestic, Strain I	i.p.	51	2.5±0.5
" Strain II	"	26	6.25±0.7
wild	"	(See Table I)	
"	s.t.	50	6.9±0.5
Dog *(Canis familiaris)*	i.p.	9	below 16
"	s.t.	7	38 (20-50)‡
Mouse *(Mus musculus)* albino	i.p.	19	56±4
Alexandrine rat *(R. rattus subsp.)*	"	14	250 (75-450)
Guinea pig *(Cavia cobaya)*	"	11	350 (300-400)
Rabbit *(Oryctolagus cuniculus)*	", s.t.	6	above 400
Cat *(Felis libyca domestica)*	s.t.	10	500 (75-1000)
Chicken *(Gallus bankiva)*			
Barred Plymouth Rock pullets	i.p.	4	2500 (?-5000)
"	s.t.	7	4250 (2500-5000)
Monkey *(Macaca mulatta)*	i.p.	6	175 (150-200)
"	s.t.	12	4250 (3500-5000)

* ANTU was administered either "i.p." (by intraperitoneal injection, usually in olive oil suspension) or "s.t." (by stomach tube, usually suspended in 10% acacia solution).

† Whenever material could not be treated statistically an estimate of the LD_{50} is followed by the range between the highest dose observed to kill none and the lowest dose killing all.

‡ Three puppies given ANTU by stomach tube survived doses up to 75 mg/kg.

small but trapped alone and able to fend for themselves, or afforded obvious external indications of maturity such as an open vagina or large descended testes. It is moderately certain that no rat weighing less than 100 g was sexually mature, while all those weighing over 200 g were indubitably adults. The age of puberty thus fell in the weight range of 100 to 200 g; more precisely, in the range 100-150 g for females and 150-200 g for males. This last statement is based upon observations made on 11 female and 5 male wild Norway rats that were raised from birth in the laboratory, which indicated that around puberty the males of a given age are heavier than the females: between 60 and 90 days old these females weighed 100-150 g and the males 150-200 g, with considerable individual variation being found within these limits, particularly between rats of different litters. (In laboratory Norways from our colony the sex difference in weight is even larger, as 60-day-old males often weigh more than 200 g while females of that age rarely weigh more than 140 g.)

In the series of rats receiving ANTU the vagina was open in about half the females with weights between 100 and 125 g, and in all the females weighing more than 150 g, which agrees well with the above statement. No definite difference in resistance to ANTU poisoning was found between individual females with open vaginas and others in the same weight range with closed vaginas, but this external sign is admittedly not the best criterion of sexual maturity. It is therefore not yet clear whether the levelling off of the LD_{50} which occurred after puberty is directly related to sexual maturity or is rather an associated phenomenon dependent on general growth factors.

Separating the 138 rats weighing more than 300 g into males and females led to respective LD_{50} values of 7.0 and 7.4 mg/kg (Table I). Thus adult females were little if any more resistant than males, indicating the absence of a marked sex variation in response to acute ANTU poisoning.

Species Variation. The differences in susceptibility to acute poisoning with ANTU which we have found between animals of various species are shown in Table II. Norway rats are seen to be the most susceptible animals of those tested, and the only ones killed by amounts less than 10 mg/kg. The dog and the mouse were the only others killed below 100 mg/kg. An intermediate group comprising the Alexandrine rat, the

guinea pig, the cat, and probably the rabbit‡ is followed by the chicken and the Rhesus monkey, both of which were extremely resistant, at least to ANTU given by stomach tube.

It is interesting to note that in contrast to the large variations in the toxicity of thiourea to Norway rats from different sources,[5] little difference was found in the response of the same rats to ANTU. Rats from Dr. Astwood's colony in Harvard (designated Strain II) were only twice as resistant as rats from our colony (Strain I), and hardly differed at all from the wild Norways.

The mode of administering the ANTU did not influence the toxicity in rats, but in other species less ANTU was required to kill by intraperitoneal injection than when given by stomach tube. For instance monkeys died following 200 mg/kg of ANTU given intraperitoneally but withstood 17 times that amount by stomach tube, and dogs likewise showed a several-fold difference. In part this may be ascribed to emesis, which occurred fairly often in dogs, cats, and monkeys, but elimination in this manner before absorption could take place was minimized by starving the animals overnight before they received doses by stomach tube, and withholding food until 6 to 8 hours later.

The effects characteristic of ANTU poisoning in Norway rats, namely pulmonary edema and pleural effusion, were not found in some species even after the administration of fatal doses. Examination of sections from the lungs of Alexandrine rats, guinea pigs, rabbits and monkeys has not revealed edema in anything like the amounts found in the lungs of Norway rats, dogs, mice and cats.[10] It would therefore seem likely that the mechanism of action of ANTU is different in these 2 groups of animals.

Both males and females were used in most species tested (the rabbits and chickens were all females) without any striking sex difference in susceptibility becoming evident. Sex differences cannot be ruled out, however, on the basis of these limited data. An indication that an age difference exists in dogs, comparable to that observed in rats, is found in the fact that 3 puppies survived doses which would have been fatal to adult dogs.

Summary. Marked age variation in the susceptibility of wild Norway rats to acute poisoning with α-naphthyl thiourea has been found, with suckling rats about 7 times as resistant as adults. This resistance was found to decrease with increasing body weight, levelling off for adults at an LD_{50} between 6 and 8 mg/kg body weight. The difference found between adult males and females was within the limits of error and is therefore not considered significant.

Adult animals of other species also varied markedly in susceptibility to acute ANTU poisoning. Norway rats, dogs and mice were killed by amounts less than a hundred milligrams per kilogram. Alexandrine rats, guinea pigs, and cats required several hundred milligrams, while chickens and monkeys survived doses of several grams per kilogram body weight. Large amounts of pulmonary edema were found only in Norway rats, dogs, mice and cats.

‡ McClosky and Smith[4] found an LD_{50} for rabbits of about 1000 mg/kg.

[10] Latta, H., unpublished results.

15359

Bactericidal Action of Streptomycin.

DOROTHY HAMRE, GEOFFREY RAKE, AND RICHARD DONOVICK.
From the Squibb Institute for Medical Research, New Brunswick, N. J.

Streptomycin,[1] which inhibits the growth of a variety of gram positive, gram negative and acid fast microorganisms[1-7] has been found by Waksman and Reilly[8] to be bactericidal for *Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris*. According to Wallace

and co-workers,[9] the bactericidal action of streptomycin on *Staphylococcus aureus* and *Eberthella typhosum* was influenced by the culture medium. Donovick and Rake[10] found that 0.056 units per ml of streptomycin inhibited the growth of 1000 cells per ml of *Klebsiella pneumoniae* in 0.75% tryptone broth and that the minimal inhibiting dose varied with the lot and the concentration of tryptone. The present study of the bactericidal action of streptomycin was made on *K. pneumoniae*,* which is used in this laboratory for the bio-assay of streptomycin.[11]

In order to follow the bactericidal action of streptomycin on this organism, 6 ml of a diluted 6-hour culture containing about 5 million cells per ml were mixed with 0.1 ml of streptomycin and incubated at 37°C. Plate counts, made at intervals in 2% tryptone 0.2% glucose agar, are given in Table I. All figures for bacterial counts are averages of 3 plates.

Comparison of the results in yeast beef broth with those in tryptone broth illustrates the effect of culture medium on the activity of streptomycin. While 2 units per ml in yeast beef broth retarded growth slightly, 1 unit per ml in tryptone broth produced a 500,000-fold reduction in count in one hour. Although different preparations of streptomycin were used, this could not account for these results, because repeated experiments in the same medium have failed to show any difference between the activity of pure and impure streptomycin.

The bactericidal effect of 1.0 unit of streptomycin per ml in tryptone broth was evident after 20 minutes incubation. On the other hand, 0.25 unit per ml caused no reduction in count during the first hour of incubation, but, following this induction period, the count dropped 60,000-fold in 3 hours, and after 24 hours incubation, rose to about one-seventh of that in the control tube. This rise in count, which also occurred in tryptone broth containing 0.5 unit per ml of streptomycin, was contrary to expectations because 1000 cells per ml in tryptone broth are inhibited by 0.056 unit per ml. Two possible explanations for the rise in count are: (1) that during the 3-hour incubation period in the presence of large numbers of cells, the streptomycin was used up, so that the concentration fell below the minimal inhibitory level; or (2) that the cells surviving at the end of 3 hours incubation with streptomycin were more resistant to its action. Experiments were set up to test the latter hypothesis. Plate counts were made in 0.75% tryptone agar with and without streptomycin. Results of these experiments are given in Fig. 1.

Although there were a few cells in the control tube resistant to 1.0 units and 3.0 units of streptomycin, the proportion of resistant to total cells in this tube remained small throughout the experiment. However, in the tube containing 0.25 unit of streptomycin per ml, the number of resistant cells in comparison to total cells, which was small after 3 hours incubation, became almost equal after 6 hours incubation and remained so through 12 and 16 hours incubation. (Exp. 2, Fig. 1). In a third experiment, when the concentration of streptomycin in the agar was increased to 4.9 and 49 units per ml, almost all the organisms surviving the bactericidal

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2 Schatz, A., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 244.

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4 Robinson, H. J., Smith, D. G., and Graessle, O. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 226.

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9 Wallace, G. I., Rhymer, I., Gibson, O., and Shattuck, M., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 127.

10 Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

* American Type Culture Collection No. 9997.

11 Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

TABLE I.
Bactericidal Action of Streptomycin on Multiplying Cultures of *Klebsiella pneumoniae*. (No. of Organisms per ml × 1000).

Incubation time	Yeast beef broth Units per ml impure streptomycin 504			
	4	3	2	0
0				6430
2 hr	17.6	>3000	46300	84600
3 "	0.8	2120	127000	212000
4 "	0.1	154	443000	836000
23 "	820000.	865000	481000	1,150000
	0.75% tryptone broth Units per ml pure streptomycin M208E			
	1.0	0.5	0.25	0
0				6760
20 min	4000.	7000.	7360.	6800
40 "	7.16	3760.	8030.	9700
1 hr	0.01	50.3	>3000.	16200
2 "	0.00	0.04	>3.0	75300
3 "	0.00	0.01	0.08	195000
24 "	0.00	122000.	109000.	753000
0				7250
1 hr		12.0	4580.	21900
2 "		0.03	1.18	67400
3 "		0.02	0.00	144000
4 "		0.03	0.07	145000
28 "		136000.	61400.	258000

effect of 0.25 unit per ml in broth after 12 hours incubation grew in plates containing 4.9 units per ml, but none grew on plates containing 49 units per ml. Apparently, then, this resistance which was acquired by this organism, or was a result of selection, is not of a high order of magnitude.

To determine whether or not this resistance was a temporary characteristic, transplants were made in the third experiment from the tube containing 0.25 unit per ml in broth after 0 hours and 16 hours incubation, to tubes containing 0.0 unit, 0.07 unit, 0.35 and 1.75 units of streptomycin per ml of tryptone broth. Serial subcultures were made after 24 hours incubation from the tube containing no streptomycin to tubes with and without streptomycin. The results of these experiments are given in Fig. 2 Three things are apparent: (1) After 16 hours incubation in broth containing 0.25 unit per ml, the organisms grew in broth containing 5 times as much streptomycin as the minimal inhibiting dose for the same culture after 0 hours. This was to be expected, since at that time the plate count in agar containing 4.9 units of streptomycin per ml was almost equal to that in agar without streptomycin (Fig. 1). (2) The amount of streptomycin in broth to which these organisms were resistant after 16 hours incubation was much less than that in agar (0.35 unit per ml compared to 4.9 units per ml, Fig. 1 and Fig. 2); (3) after 3 subcultures in the absence of streptomycin, the 2 cultures retained their respective characteristics, an indication that a persistent change in the resistance of the organism had occurred.

Some evidence was also obtained for the appearance of resistance in *Klebsiella pneumoniae* by repeated subculturing in tryptone broth containing streptomycin. After 2 serial subcultures in 0.35 unit of streptomycin per ml, the resistance of the organisms taken originally from broth containing 0.25 unit per ml after 16 hours incubation rose from 0.35 unit per ml to 1.75 units per ml. (Fig. 2). However, this 5-fold increase in resistance is slight in comparison with the increase in resistance of gonococci and meningococci from about 40 units per ml to 75,000 units per ml which Miller and Bohn-

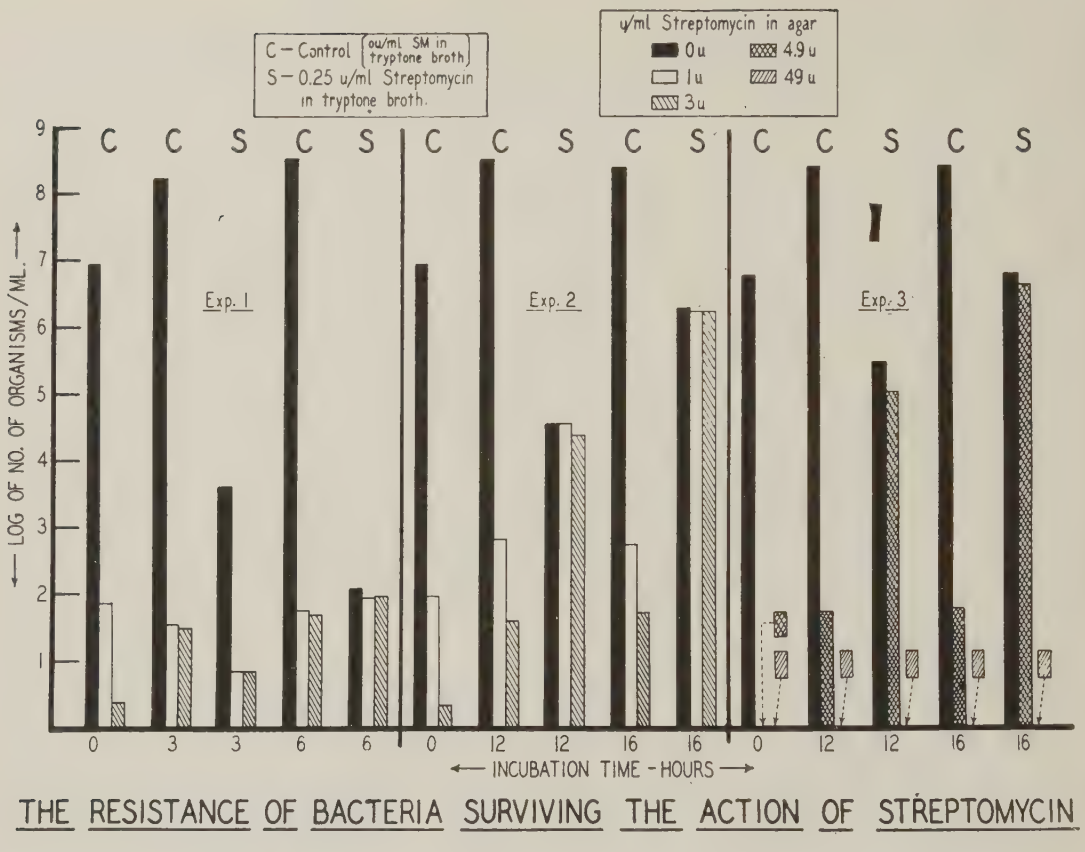

Fig. 1.

TABLE II.
Bactericidal Action of Streptomycin on Washed Cultures of *Klebsiella pneumoniae*.
(No. of Organisms per ml × 1000).

Incubation time	Units per ml pure streptomycin M208E			
	30	15	7.5	0
0				5760
20 min	1100.	5300.	5530.	6100
40 ''	3.55	616.	5000.	5730
1 hr	.02	10.4	1983.	5430
2 ''	<.01	.023	12.9	4900
3 ''	<.01	.013	.066	7300
	15	7.5	3.75	0
0				5700
20 min	5600.	6100.	6100	5230
40 ''	2590.	4230.	5830	5430
1 hr	220.	3360.	5600	5560
2 ''	.00	108.	2050	7960
3 ''	.00	.00	172	7800
		3.78	1.89	0
0				4530
2 hr		5260	5860	5700
4 ''		3240	6800	7600
6 ''		443	6130	8130

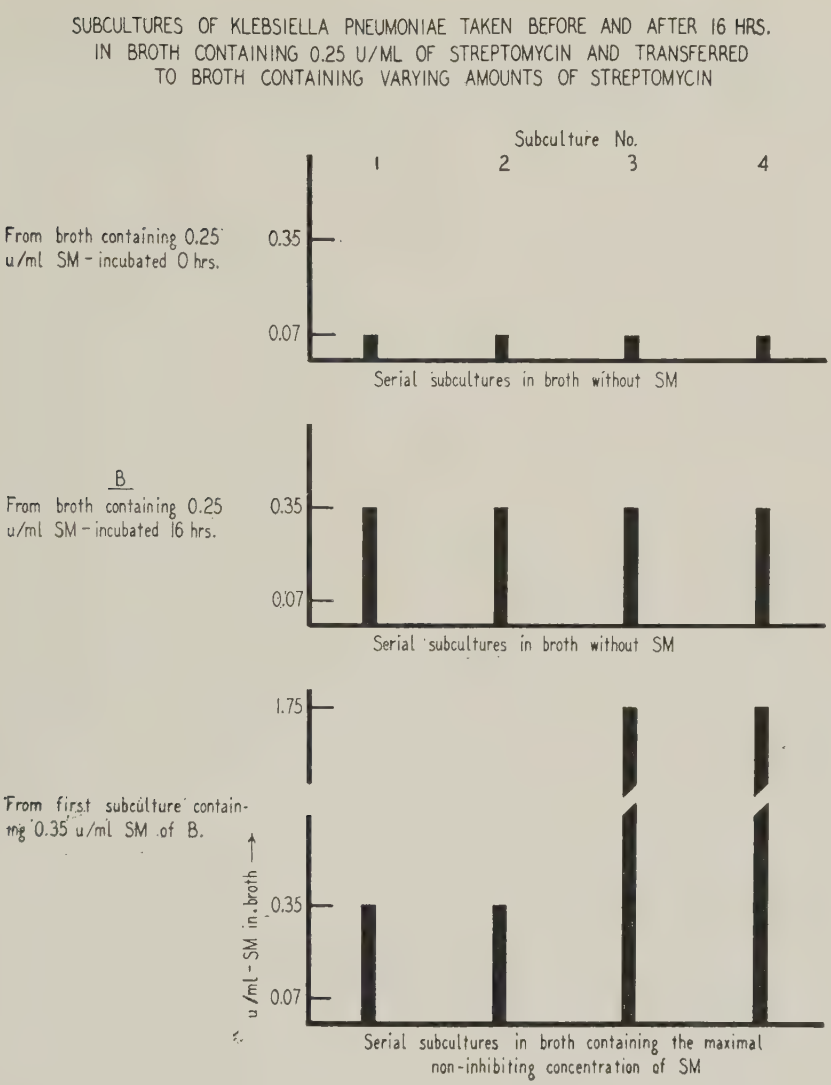

Fig. 2.

hoff[12] were able to obtain by serial transfer on agar containing streptomycin.

The bactericidal action of streptomycin on non-multiplying cultures of *K. pneumoniae* was next investigated. A 6-hour culture was washed 3 times by centrifuging with Ringer's solution, resuspended in Ringer's solution to give approximately 10 million cells per ml and mixed with an equal volume of streptomycin in M/15 phosphate buffer pH 6.8. During incubation at 37°C plate counts were made in 2% tryptone 0.2% glucose agar. Results of average counts of 3 plates are given in Table II.

Thirty units per ml reduced the number of viable cells greater than 5-fold after 20 minutes incubation, 3.75 units per ml caused a slight drop in count after 3 to 6 hours incubation, but 1.89 units per ml had no effect. These results indicate that the mode of action of streptomycin differs from that of penicillin, which had no action on

[12] Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1945, **130**, 485.

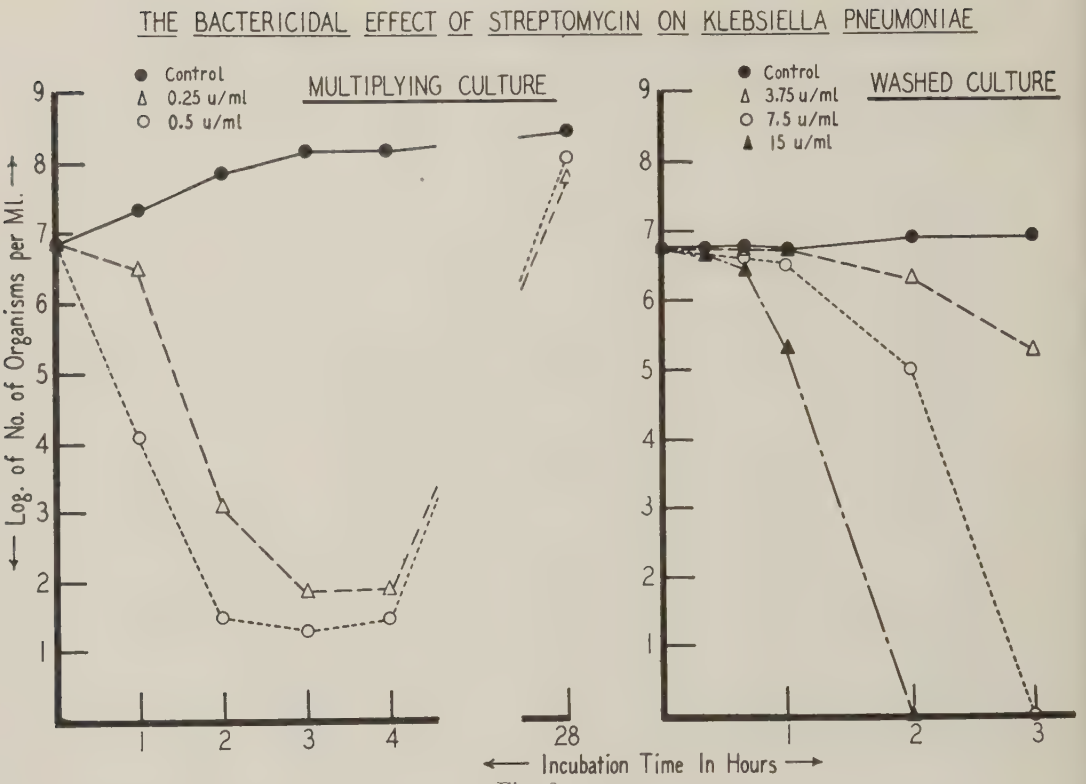

Fig. 3.

a washed culture of *Staphylococcus aureus*, Heatley strain, even at 1000 units per ml.[13]

Because the amount of streptomycin required to produce a drastic drop in the number of viable cells in a washed, non-multiplying culture is much greater than that required for a multiplying culture (Fig. 3), it seems probable that the mode of action of streptomycin is different for washed, non-multiplying bacteria.

It will be recalled that with multiplying cultures almost all the organisms surviving 6 hours incubation with 0.25 unit of streptomycin per ml were more resistant to streptomycin. In experiments with washed cultures, incubated with 7.5 units per ml and plated in agar containing 1.0 and 3.0 units of streptomycin per ml, no evidence was obtained to indicate that the organisms surviving 3 hours or 5 hours incubation were more resistant than the control. This also favors the theory that the mode of action of streptomycin on washed organisms is different from that on multiplying cultures.

In view of the bactericidal action of streptomycin on washed cultures of *K. pneumoniae*, it was of interest to determine whether or not streptomycin had any action on washed spores of a susceptible species. It was found that 43,100 units per ml in buffered Ringer's solution had no effect during 6 hours or 24 hours incubation with washed spores of an unidentified gram positive bacillus (*Bacillus sp.* No. 290). Multiplying cultures of this organism were very sensitive to streptomycin, being inhibited by as little as 0.013 unit per ml in tryptone broth containing 1500 cells per ml.

Summary. Streptomycin was bactericidal for both multiplying and non-multiplying cultures of *K. pneumoniae*, but not for washed spores of *Bacillus sp.* No. 290. Organisms surviving the bactericidal effect of 0.25 unit of

[13] Todd, E. W., *Lancet*, 1945, **1**, 74.

streptomycin per ml after 6 hours incubation were more resistant to its action. This resistance persisted through 4 subcultures in the absence of streptomycin and could be increased 5-fold by serial transfers in broth containing streptomycin. Washed organisms surviving after 5 hours incubation with 7.5 units of streptomycin per ml were not more resistant.

15360

A Procedure for Testing Sterility of Concentrated Streptomycin Solutions.

GEOFFREY RAKE AND RICHARD DONOVICK.

From the Squibb Institute for Medical Research, New Brunswick, N. J.

Since the use of streptomycin as a therapeutic agent is rapidly increasing it is important to be able to test the sterility of highly concentrated solutions of this antibiotic. In such sterility tests it is, of course, a prerequisite that the sample of streptomycin taken be of sufficient volume to be representative of the lot of streptomycin involved. As the bacteriostatic levels of streptomycin, at least in the case of many organisms, differ greatly from bactericidal concentrations[1] the addition of such an adequate sample of streptomycin to the sterility test broth may result in inhibition of growth of any viable cells which may be present. Hence there is need for a method of inactivating streptomycin without destroying any living organisms present.

In order to test the sterility of streptomycin solutions in this laboratory, a procedure has been devised which incorporates the inactivation of streptomycin with semicarbazide[2,4] and the use, as the culture medium, of thioglycolate broth which interferes with streptomycin activity.[3]

Of the carbonyl reagents which inactivate streptomycin, semicarbazide was chosen because it is one of the least bacteriostatic members of this group (Table I) and is readily soluble in water. Thiosemicarbazide is also relatively low in bacteriostatic activity but is much less soluble.

TABLE I.
Comparative Sensitivity of Various Organisms to Streptomycin and to Certain Carbonyl Reagents.

	Growth inhibiting concentration in 1% tryptone broth					
Organism	Streptomycin mg/ml	HA-HCl mg/ml	HZ-H_2O mg/ml	SC-HCl mg/ml	TSC mg/ml	Ratio SC-HCl/streptomycin
K. pneumoniae (A.T.C.C. No. 9997)	0.000055*	0.0088	0.0068	0.073	0.116	1325
E. coli No. 33	0.00012*	0.016	0.010	0.064	0.088	533
Staph. aureus (Heatley)	0.000049*	0.018	0.017	>0.54	0.017	>11000
B. subtilis No. 558	0.000056*	0.012	0.012	0.48	0.23	8570
Bacillus sp. No. 290	0.000013*	0.010	0.0073	0.089	0.15	6840

* Based on pure streptomycin base as having 1000 units per mg.
HA-HCl = hydroxylamine hydrochloride.
HZ-H_2O = hydrazine hydrate.
SC-HCl = semicarbazide hydrochloride.
TSC = thiosemicarbazide.

1 Hamre, D., Rake, G., and Donovick, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 25.

2 Brink, N. G., Kuehl, F. A., and Folkers, K., *Science*, 1945, **102**, 506.

3 Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

4 Donovick, R., Rake, G., and Fried, J., *J. Biol. Chem.*, in press.

Method. Materials required for inactivation of streptomycin solution containing 30,000 units per ml ± 10%:

1. Semicarbazide-hydrochloride solution: ca. 0.06 g per ml. Sterilization through U. F. fritted glass filter is the preferred method. The sterile solution is stored at 4°C.

2. Potassium acetate solution: ca. 0.05 g per ml. Solution is sterilized by autoclaving, 10 lb pressure for 10 minutes.

Procedure for inactivation of streptomycin. To each ml of streptomycin solution to be inactivated is added 1 ml of the potassium acetate solution and then 1 ml of semicarbazide-hydrochloride solution, observing aseptic precautions. The mixture is incubated at ca. 25°C (room temperature) for 24 hours.

After room temperature incubation, the inactivation mixture is tested for sterility in freshly prepared thioglycolate broth, using at least 30 ml of broth for each ml of inactivation mixture to be tested. The inoculated broth is incubated at 37°C for at least 24 hours.

Discussion. It has been shown that 2 γ of semicarbazide hydrochloride per γ (or unit) of streptomycin will cause 98% inactivation at pH 5.3.[4] Since the semicarbazide-hydrochloride solution is extremely low in pH, potassium acetate is first added to the streptomycin solution to bring the final mixture to the desired pH.

To justify the use of this method of inactivation of streptomycin as a step prior to sterility testing it must be shown that any organism resistant to 30,000 units of streptomycin per ml is also resistant to the concentration of semicarbazide present in the inactivation mixture. This question has been approached in 2 ways: Thus far we have found no vegetative cells resistant to such concentrations of streptomycin but have compared the sensitivities of a number of species to this antibiotic with their sensitivities to several carbonyl reagents at much lower concentrations of both substances. The results of such studies with 5 species are given in Table I. The second approach to this problem is based on the finding that spores of some species of bacteria are extremely re-

TABLE II. Recovery of *Bacillus sp.* No. 290 from Streptomycin Inactivated by Semicarbazide.

Tube No.	Mixture incubated at 25°C, 24hr: Spore suspension* ml	Streptomycin† ml	Semicarbazide-hydrochloride‡ ml	Potassium acetate§ ml	H_2O	Mixture diluted in thioglycolate broth, incubated at 37°C, 48 hr: Growth after dilution in broth 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	0.3	0.3	0.3 (undil.)	0.3	—	—	+++	+++	+++	+++	—
2	0.3	0.3	0.3 (½)	0.3	—	—	+++	+++	+++	+++	—
3	0.3	0.3	0.3 (⅓)	0.3	—	—	+++	+++	+++	+++	—
4	—	0.3	0.3 (undil.)	0.3	0.3	—	+	+	++	++	—
5	0.3	—	0.3 ,,	0.3	0.3	—	+	+	++	++	—
6	0.3	0.3	—	—	0.6	—	—	—	—	—	—
7	0.3	—	—	0.3	0.6	+	+	—	—	—	—
8	—	0.3	—	—	0.9	—	—	—	—	—	—
9	—	—	0.3 ,,	0.3	0.9	—	—	—	—	—	—
10	—	—	—	—	0.9	+	+	—	—	—	—
11	0.3	—	—	—	1.2	—	—	—	—	—	—
12	—	—	—	—	—	—	—	—	—	—	—

* Spore suspension contained ca. 1,000,000 spores per ml.
† Streptomycin solution = 28,700 units per ml = 0.0287 g per ml.
‡ Semicarbazide-hydrochloride solution = 0.0862 g per ml in undiluted solution.
§ 5% potassium acetate solution.
+ = growth; — = no growth.

sistant to the germicidal action of streptomycin even though the growth of their vegetative forms is inhibited by very low concentrations of streptomycin in broth.[1] The spores of such an organism (*Bacillus sp.* No. 290) were added to a solution containing 28,700 units of streptomycin per ml and the streptomycin solution then was inactivated in the manner described. The results of a typical experiment are tabulated in Table II.

It will be seen in Table I that the growth-inhibiting concentration of semicarbazide ranges from 533 to >11,000 times greater than the inhibiting concentrations of streptomycin for the organisms tested.

Ability of spores of Bacillus sp. No. 290 to resist the action of semicarbazide during the inactivation of streptomycin. Various mixtures of spores, streptomycin, semicarbazide-hydrochloride and potassium acetate were prepared as shown in Table II and incubated at 25°C for 24 hours. Each mixture was then diluted by 10-fold steps in freshly prepared thioglycolate broth, the broth dilutions were incubated at 37°C, and read for growth after 24, 48 and 96 hours incubation. Little change occurred after 48 hours incubation; therefore only the 48-hour readings are recorded in the table.

It is shown in Table II that the streptomycin-spore suspension mixture gave no growth until diluted 10^{-4} in thioglycolate broth, whereas growth occurred at 10^{-2} in mixtures containing 3, 2 and 1 γ of semicarbazide hydrochloride per γ of streptomycin. When spores were added to semicarbazide-hydrochloride and diluted in thioglycolate broth, growth again occurred in the 10^{-2} dilution. Hence under the conditions of the experiment presented, streptomycin, containing viable cells, when treated with the specified carbonyl reagent yielded growth at 1/100th the dilution required to obtain growth from the original streptomycin-spore suspension mixture.

Summary. 1. Details are given for a method of inactivating streptomycin with semicarbazide-hydrochloride in order to test the sterility of concentrated streptomycin solutions. It has been shown that although several carbonyl reagents inhibit bacterial growth, it requires from 533 to >11,000 times more semicarbazide-hydrochloride (one of the least toxic of the group) to cause this inhibition than is required of streptomycin.

2. Spores of *Bacillus sp.* No. 290, when added to a streptomycin solution containing 28,700 units per ml, were able to grow out when diluted 10^{-4} in thioglycolate broth. When a similar spore suspension-streptomycin mixture was treated with semicarbazide-hydrochloride (3, 2 or 1 γ carbonyl reagent per unit (or γ) of streptomycin) and then diluted in thioglycolate broth, growth occurred at a 10^{-2} dilution.

15361

Buffers in the Range of pH 6.5 to 9.6.*

GEORGE GOMORI.
From the Department of Medicine, The University of Chicago.

In the pH range between 6.5 and 9.6, the buffers generally used have been phosphate, barbital,[1] ammonium salts and carbonate.[2] Among these, phosphate and carbonate are incompatible with Ca salts; ammonium salt buffers are not entirely stable; barbital, on account of its low solubility, can be prepared in low concentrations only and, in addition, inhibits certain enzyme systems.[3] Mertz and Owen[4] have suggested the use of imidazole as a buffer in the physiologic pH range, com-

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research at the University of Chicago.

1 Michaelis, L., *J. Biol. Chem.*, 1930, **87**, 33.

2 Delory, G. E., and King, E. J., *Bioch. J.*, 1945, **39**, 245.

3 Quastel, J. H., and Wheatley, A. H. M., *Proc. Roy. Soc. B.*, 1932, **112**, 60; *Bioch. J.*, 1934, **28**, 1251.

TABLE I.
pH Values of Buffers at 23°C and 37°C.

0.1 N HCl cc	Collidine		Tris(hydroxymethyl)-aminomethane		2-amino-2-methyl-1,3-propanediol	
	23°C	37°C	23°C	37°C	23°C	37°C
*5.0	8.35	8.28	9.10	8.95	9.72	9.62
7.5	8.18	8.10	8.92	8.78	9.56	9.45
10.0	8.00	7.94	8.74	8.60	9.38	9.27
12.5	7.88	7.80	8.62	8.48	9.26	9.15
*15.0	7.77	7.70	8.50	8.37	9.15	9.03
17.5	7.67	7.60	8.40	8.27	9.05	8.94
20.0	7.57	7.50	8.32	8.18	8.96	8.85
22.5	7.50	7.40	8.23	8.10	8.87	8.76
*25.0	7.40	7.32	8.14	8.00	8.78	8.67
27.5	7.30	7.23	8.05	7.90	8.70	8.58
30.0	7.22	7.14	7.96	7.82	8.60	8.50
*32.5	7.13	7.05	7.87	7.73	8.50	8.40
35.0	7.03	6.95	7.77	7.63	8.40	8.30
*37.5	6.92	6.84	7.66	7.52	8.30	8.20
40.0	6.80	6.72	7.54	7.40	8.18	8.07
42.5	6.62	6.54	7.36	7.22	8.00	7.90
*45.0	6.45	6.37	7.20	7.05	7.83	7.72

patible with Ca; however, its high cost is almost prohibitive.

Three new buffers: 2,4,6-collidine, tris(hydroxymethyl)-aminomethane and 2-amino-2-methyl-1,3-propanediol, are suggested for the use in the pH range between 6.5 and 9.6. They are quite soluble, do not precipitate Ca salts, and are low in price. They were found to be stable at room temperature for a period of over 3 months. Collidine and Tris(hydroxymethyl)-aminomethane, to be used in the pH ranges between 6.5 and 8.3, and between 7.2 and 9.0, respectively, were tested by Dr. E. S. Guzmán Barrón for their effect on the O_2 uptake of rat kidney slices in the presence of 0.01 M pyruvate. The concentration of the buffers was 0.02 M, phosphate buffer being used as a control. The results with the different buffers were all well within the limits of experimental error, thus showing complete lack of inhibitory action. Tris(hydroxymethyl) - aminomethane and 2-amino-2-methyl-1,3-propanediol (range, pH 8.0 to 9.7) were tested for their effect on alkaline phosphatase at pH 9.1, 0.005 M glycerophosphate being used as a substrate. Barbital and Delory and King's[2] carbonate buffers served as controls. Again, no inhibitory effect was noted.

The pK_b values of the new buffer substances were determined by the electrometric determination of the pH of their half-neutralized 0.05 M solutions at 23°C and 37°C. The apparatus used was a Leeds and Northrup potentiometer with glass and calomel electrodes. Phthalate buffer served as a standard.

1. 2,4,6-collidine (s-collidine).† Colorless liquid; pH 7.4 at 23°C; 7.32 at 37°C, pK_b 6.6 and 6.68, respectively.

2. Tris(hydroxymethyl) - aminomethane.‡ Colorless crystals; pH 8.14 at 23°C; 8.00 at 37°C; pK_b 5.76 and 6.0, respectively.

3. 2-amino-2-methyl-1,3-propanediol.‡ Colorless, somewhat hygroscopic crystals; pH 8.78 at 23°C; 8.67 at 37°C; pK_b 5.22 and 5.33, respectively.

The pH values of 0.05 M buffer mixtures, obtained by mixing 25 cc of a 0.2 M solution of the bases (collidine, 2.64 cc in 100 cc; tris(hydroxymethyl)-aminomethane, 2.43 g in 100 cc; 2-amino-2-methyl-1,3-propanediol, 2.1 g in 100 cc) with varying volumes of 0.1 N HCl and diluting the mixtures to the final volume of 100 cc, are given in Table I. The values marked with an asterisk were determined by potentiometric measurement, all the other ones were interpolated by calculation. The effect of salts on these values was not determined.

[4] Mertz, E. T., and Owen, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 204.

† Obtainable from the Eastman Kodak Co., Rochester, N. Y.

‡ Obtainable from the Commercial Solvents Corporation, 17 East 42nd Street, New York.

15362 P

Therapeutic Effectiveness of Single Oral Doses of Penicillin.

H. J. White, M. E. Lee, and C. Alverson. (Introduced by Raymond N. Bieter).

From the Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Conn.

Very few chemotherapeutic agents are effective when administered as single oral doses in acute bacterial infections. Robinson, Smith and Graessle[1] reported 80% survival with a batch of crude streptomycin, using a single oral dose of about 1500 mg per kilo, in an acute *Salmonella* infection in mice.* These investigators also found that a single oral dose of about 500 mg per kilo of crude streptothricin (assumed potency 300 units per mg) saved 76% of mice in a similar infection.[2] Finally, each of several analogues of pantothenic acid, administered as a single oral dose of 500 mg per kilo, has produced 100% survival in an acute hemolytic streptococcal infection in mice.

It is the purpose of the present communication to report preliminary results of a study of the therapeutic effectiveness of single oral doses of penicillin.

Methods. The penicillin powder used in this work was labelled "Crude, Penicillin G, pH 5.0 salt." It was supplied by Lederle Laboratories, Pearl River, N. Y. Its potency was determined as 650 *Staphylococcus* units per mg. Thus, on the basis of 1667 units per mg of pure penicillin G, the purity of the powder was considered to be approximately 39%.

The untreated experimental streptococcal infection is characterized by rapidly developing septicemia uniformly terminating in death within 48 hours. Treated animals alive on the 21st day after infection are considered to be completely protected.

Results. The therapeutic effect which can be obtained with a single oral dose of penicillin is shown in Table I. It is evident that a single dose of only 25 mg (equivalent to 16,000 to 17,000 units) per kilo of mouse produced a marked effect (97% survival).

TABLE I.

Therapeutic Effectiveness of Single Oral Doses of Penicillin.

Organism: *Hemolytic streptococcus;* Group A; strain C203.
Mice: Vanderwerken; 18-22 g.
Infection: Intraperitoneal; 0.5 cc of a 10^{-5} broth dilution of a 6-hour TSP-blood broth culture; 4000 ± 400 organisms.
Treatment: Penicillin powder containing 650 units per mg; single dose by stomach tube immediately after infection.

Single dose mg/kg	Survival on 21st day after infection alive/total No.	Dose calculated as pure penicillin G mg/kg
4000	10/10	1600
2000	10/10	800
1000	10/10	400
500	10/10	200
250	30/30	100
200	30/30	80
150	30/30	60
100	30/30	40
50	30/30	20
25	29/30	10
Untreated controls	0/40	—

Discussion. The fact that 97% survival was obtained with crude penicillin powder (650 units per mg) administered orally as a single dose of 25 mg per kilo suggests clinical applications for streptococcal infections. Thus, an equivalent dose for a 60-kilo patient would be 1.5 g; expressed as pure penicillin G, this dose would be only 0.6 g.

[1] Robinson, H. J., Smith, D. G., and Graessle, O. E., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 226.

* The dosage reported was 3000 units. The dose given above was calculated on the assumptions that the streptomycin sample contained 100 units per mg and that the average mouse weight was 20 g.

[2] Robinson, H. J., and Smith, D. G., *J. Pharm. and Exp. Therap.*, 1944, **81**, 390.

Such doses appear to be "massive" only when expressed as "units." The advantages of therapeutic administration of penicillin in a single oral dose, or in oral doses given once or twice a day, are obvious.

Further work is in progress to determine the effectiveness of single oral doses of different lots of penicillin in the streptococcal and other infections in mice.

Summary. Survival of 97% was obtained in a streptococcal infection in mice with crude penicillin powder containing 650 units per mg, administered in a single oral dose of 25 mg per kilo.

15363 P

Adenosine Triphosphatase Activity and Weight of Corpora Lutea During Reproductive Cycle of the Rat.*

Clyde Biddulph, Roland K. Meyer, and W. H. McShan.

From the Department of Zoology, University of Wisconsin, Madison.

Adenosine triphosphate (ATP) and the ATP-splitting enzyme, adenosine triphosphatase (ATP-ase), have been found in tissues other than muscle,[1] and evidence has been presented that the ATP system links the energy-requiring reactions of tissues with the energy-yielding reactions.[2] These results suggested an investigation of the ATP-ase activity of ovarian structures since these tissues undergo rapid changes in growth and function during which large amounts of energy must necessarily be involved.

Materials and Methods. The method of DuBois and Potter[1] was used in determining the ATP-ase activity of corpora lutea and remaining ovarian tissue of female rats during estrus, diestrus, pseudopregnancy and lactation at the intervals indicated in Table I. This method measures the amount of inorganic phosphorus released by the enzyme of homogenized tissue when ATP is used as substrate in a buffered solution (pH 7.4) containing calcium. The corpora lutea were dissected from the ovaries using sharp-pointed scalpel blades and spectacle loupes. The identification of the corpora of pregnancy and corpora of the post-partum ovulation throughout lactation was based on the differences in size, shape and color. That these criteria were adequate was confirmed by marking the corpora of pregnancy with fine silk thread. The corpora and ovarian residue were weighed and homogenized prior to adding to the incubation mixture. Each ATP-ase value in the table is the average of 9 determinations (3 for each of 3 rats), and each weight is the average of the corpora lutea obtained from 3 rats.

Results and Discussion. In diestrus there was a slight increase in the ATP-ase of the corpora lutea and ovarian residue above the estrus level. The average weight of the diestrous corpora was essentially the same as that found in estrus. During pseudopregnancy the weight of the corpora increased somewhat from the estrus level, but the ATP-ase activity did not change significantly. As pseudopregnancy progressed a definite decrease in the value of the ovarian residue was noted. At 11 days estrus was imminent, and the results suggest an inverse relationship between estrogen level and ATP-ase of the extra-luteal tissues.

The ATP-ase activity of the corpora lutea during pregnancy, when calculated on a per mg of tissue basis, did not show marked variation despite the fact that the average weight of the corpora lutea progressively increased. Late in pregnancy an increase in activity was found which continued until the

* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

1 DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **150**, 185.

2 Potter, V. R., *J. Cell. and Comp. Physiol.*, 1945, **26**, 87.

TABLE I.

Tissue	Estrus	Diestrus	Pseudopreg.			Stage Pregnancy				Lactation					
		2*	4	7	11	4	7	11	15	20	4	7	11	15	20	22	23
Avg wt. (mg) Corpora lutea	1.2	1.1	1.5	1.6	—	1.5	1.4	2.1	4.0	4.5	2.4†	1.6	0.7	0.6	0.5	0.4	0.5
											1.5	1.8	1.5	1.7	2.1	2.2	2.9
Avg ATP-ase Units‡ Corpora Lutea	7.8 ±.47§	9.8 ±.60	7.2 ±.32	7.9 ±.54	8.1 ±.40	7.4 ±.15	7.8 ±.31	7.5 ±.17	5.8 ±.81	9.2 ±.17	15.9† ±.13	16.6 ±.76	24.9 ±.95	20.6 ±.43	16.5 ±.87	16.4 ±.88	15.4 ±1.3
											5.5 ±.24	7.5 ±.65	7.8 ±.71	6.7 ±.28	9.1 ±.47	12.3 ±.57	9.8 ±.40
Avg ATP-ase Units‡ Ovarian residue	13.6 ±.54	15.7 ±.27	18.0 ±.25	17.6 ±.84	11.2 ±.57	13.5 ±.74	19.7 ±.66	13.8 ±.22	14.3 ±.82	12.6 ±.20	16.3 ±.64	17.7 ±.43	19.8 ±1.3	16.8 ±.59	15.0 ±.23	16.0 ±.78	14.7 ±.83

* Days. † First line, corpora of pregnancy which persist during lactation; second line, corpora lutea of the post-partum ovulation.
‡ A unit is the quantity of ATP-ase which liberates 1 γ of inorganic phosphorus from ATP in 15 minutes at 37°C. The values are expressed as units per mg of fresh tissue,[1] each value being the average of 9 determinations (3 for each of 3 rats). § Standard error of the mean.

11th day of lactation, during which time the average weight of the corpora lutea decreased abruptly.

During the first 20 days of lactation the ATP-ase activity of the corpora lutea of the post-partum ovulation paralleled closely the activity of the corpora of pregnancy during gestation. The increased ATP-ase after day 20 was correlated with decreasing lactation and reappearance of estrous cycles.

When the above results are calculated on the basis of change per corpus luteum rather than change per mg of tissue, the weight and the ATP-ase activity increase at practically identical rates during the periods of gestation in which there is active progesterone secretion.[3,4] The same relationship holds for the corpora of the post-partum ovulation during the first 20 days of lactation. Immediately before parturition and late in lactation the relationship changes so that the rate of change in ATP-ase activity exceeds the weight change rate. Following parturition both the weight and ATP-ase activity decrease, but the latter lags considerably behind the former. These results indicate a parallel increase in size and ATP-ase activity of the functional lutein cell, which suggests, together with other evidence linking the ATP system with lipid metabolism,[5,6] that ATP-ase may be involved in the production of progesterone by the functional corpus luteum.

The enzyme activity of the ovarian residue during pregnancy increased to a high value on day 7, then decreased significantly on day 11, and remained approximately unchanged until after parturition. Following littering a maximum was reached on day 11 of lactation.

We have also studied the succinic dehydrogenase activity of lutein and ovarian tissues throughout pregnancy and lactation[7] to

[3] Atkinson, W. B., and Hooker, C. W., *Anat. Rec.*, 1945, **93**, 75.

[4] Laqueur, G. L., and Koets, P., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 239.

[5] Lardy, H. A., Hansen, R. G., and Phillips, P. H., *Arch. Biochem.*, 1945, **6**, 41.

[6] Lehninger, A. L., *J. Biol. Chem.*, 1945, **157**, 363.

[7] Unpublished data.

extend previous studies of this enzyme system in these tissues.[8] In these studies it was also found that the dehydrogenase activity varies with the stage of the reproductive cycle, there being a steady increase in the QO_2 of the corpora lutea of pregnancy until midterm after which this level is maintained until parturition. During lactation the value declines rapidly to the early pregnancy level and remains there throughout 20 days of suckling. The QO_2 of the corpora lutea of the post-partum ovulation increases until day 20 of lactation when the highest value obtained from any ovarian tissue is reached. The QO_2 of the ovarian residue does not vary markedly during pregnancy or lactation.

Summary. The ATP-ase activity and weight of lutein and ovarian tissue were determined during estrus, diestrus, pseudopregnancy, pregnancy and lactation. The results indicate that in general the enzyme activity per unit weight is lower in functional corpora lutea than in apparently non-functional corpora. The weight and enzyme activity per corpus luteum increased and decreased at approximately the same rate during pregnancy and lactation. The enzyme activity of ovarian tissue remaining after the removal of the corpora lutea was greatest on the 4th day of pseudopregnancy, 7th day of pregnancy and 11th day of lactation.

[8] Meyer, Roland K., McShan, W. H., and Erway, Wilma F., *Endocrinology*, 1945, **37**, 431.

15364

Effect of Prolonged Wakefulness on the Urinary Excretion of 17-Ketosteroids.*

DAVID B. TYLER, WALTER MARX, AND JOSEPH GOODMAN.

From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, Calif.

Selye[1] has shown that a number of different types of stress cause a stimulation of the adrenal cortex. As a result of this stimulation, the adrenal secretes more substances of steroid nature, and the level of 17-ketosteroids in the urine is increased. Pincus and Hoagland[2] demonstrated such an increase in the output of 17-ketosteroids in pilots under conditions of stress produced by actual flight.†

Prolonged wakefulness may be considered a condition which causes considerable stress on the organism. If the response of the adrenal cortex to stress were a general phenomenon, it would be expected that prolonged wakefulness would induce a similar stimulation of the cortex with a resulting increase in the excretion of 17-ketosteroids.

Methods. Twelve subjects,‡ ranging in age from 18 to 33 years, were used. They were divided into 2 groups of 6 each. One group (B) received 1 grain of amytal§ every 12 hours for the first 48 hours of the sleepless period and then placebos every 12 hours there-

* The work described in this report was done under contract recommended by the CMR, between the Office of Scientific Research and Development and the California Institute of Technology.

[1] Selye, H., *Endocrinol.*, 1937, **21**, 169.

[2] Pincus, G., and Hoagland, H., *J. Aviat. Med.*, 1943, **14**, 173.

† After this MS. was completed, Pincus *et al.*[8] reported that the 17-ketosteroid excretion is reduced during sleep.

‡ Twelve conscientious objectors of the Glendora Civilian Public Service Camp volunteered for this and other experiments carried out under this project, and their cooperation was excellent.

§ Amytal was given in this experiment to determine its effect on the performance of men who might be forced to remain awake for prolonged periods of time. It was a constituent of a motion sickness preventive being considered for general use by the armed services.

TABLE I.
Total Daily Urinary 17-Ketosteroids.

	Control period, normal sleep days			Experimental period, no sleep days			
	1st	2nd	3rd	1st	2nd	3rd	4th
Group A							
Med.	—	—	—	Plac.	Plac.	Plac.	Plac.
KA	20.8	23.6	24.9	22.6	20.9	21.5	18.5
EB	17.0	18.1	15.7	14.0	16.1	14.8	17.0
BB	11.8	13.1	11.9	12.7	11.5	15.2	11.5
LC	21.1	22.7	23.4	18.6	18.4	19.0	21.2
DM	14.2	17.1	14.5	17.2	19.1	17.6	14.6
RT	19.1	19.3	19.3	20.6	16.7	10.7	13.7
Mean	(17.4)	(19.0)	(18.2)	(17.6)	(17.1)	(16.5)	(16.1)
Group B							
Med.	—	—	—	Amytal	Amytal	Plac.	Plac.
BA	17.6	21.8	19.8	18.8	14.4	17.3	17.9
MD	15.4	14.7	13.5	13.0	10.1	11.4	13.3
CE	20.7	20.7	23.7	22.9	17.8	20.6	17.9
EF	16.0	15.4	19.7	17.1	14.3	16.4	16.5
DL	10.5	12.9	12.3	14.6	12.0	9.2	12.3
TL	17.1	21.0	18.7	16.3	17.5	19.3	18.0
Mean	(16.1)	(17.8)	(18.0)	(17.1)	(14.4)	(15.7)	(16.0)

Values expressed as mg dehydro-iso-androsterone.

after. The other group (A) received placebos throughout. All received the same food and participated in the same activities.‖

Urine was collected, first as a control under normal conditions of sleep, for 3 days prior to the experiment, and during the sleepless period of 112 hours, as follows: Each man saved his entire output for each 24-hour period in a bottle containing 10 cc of concentrated HCl. Each sample was acidified immediately after the termination of each 24-hour period with a volume of concentrated HCl equivalent to 15% of the total urine volume, and stored at 2-4°C.

Pincus' procedures for hydrolysis and extraction,[3] and the colorimetric method of Callow[4] were employed. The concentration of total 17-ketosteroids was calculated by means of a calibration curve which had been established with graded amounts of dehydro-iso-androsterone acetate. Suggestions by Talbot *et al.*[5,6] and Engstrom and Mason[7] were followed for correction of the results for the influence of non-ketonic chromogens. In 13 representative samples, the non-ketonic fraction was separated from the ketones by the use of Girard's reagent T; from the colorimeter readings obtained with these samples, a calibration curve was drawn for the correction of all other samples.

Results and Discussion. The results are given in the table, and indicate that:

Prolonged wakefulness did not change the level of the urinary 17-ketosteroids. The values for each individual of the placebo group, as well as for this group as a whole, were not significantly different for the control and the experimental periods. This is in agreement with a previous, but incomplete, study made by one of us. (D.B.T.) on 20 subjects in which prolonged wakefulness

‖ In order to keep the men awake for prolonged periods, it is necessary that they be on a program of continuous physical activity. This is particularly important after the first day. This experiment is one of many dealing with prolonged wakefulness involving almost 700 subjects. Because of the interest at the moment in 17-ketosteroid output during various conditions of stress, it seems to be desirable to report the results of this phase of the study at the present time.

3 Pincus, G., and Pearlman, W. H., *Endocrinol.*, 1941, **29**, 413.

4 Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, 1938, **32**, 1312.

5 Talbot, N. B., Butler, A. M., MacLachlan, E. A., and Jones, R. N., *J. Biol. Chem.*, 1940, **136**, 365.

6 Talbot, N. B., Berman, R. A., and MacLachlan, E. A., *J. Biol. Chem.*, 1942, **143**, 211.

7 Engstrom, W. W., and Mason, H. L., *Endocrinol.*, 1943, **33**, 229.

appeared to have no significant effect on the urinary 17-ketosteroids.

The administration of 1 grain of amytal every 12 hours during the first 48 hours of the sleepless period resulted in a slight but noticeable depression in the excretion of these substances. This was most noticeable on the 2nd day. Upon stopping the amytal administration there was a gradual rise to the pre-amytal or control values.

Total urine volumes varied greatly between individuals as well as from day to day. The amount of ketosteroid excreted appeared to be practically independent of the total urine volume.

The observations that fatigue from sleeplessness did not influence the urinary excretion of 17-ketosteroids are in contradiction to the findings of Pincus and Hoagland,[2] and Pincus, Hadidian and Yeaton.[8] It must be pointed out, however, that in our experiments the output of an entire 24-hour period was measured, while the results of Pincus and Hoagland were based on samples collected during relatively short periods of stress. Furthermore, the type of stress was different and, therefore, the results are not strictly comparable.

Summary. 1. The urinary excretion of 17-ketosteroids of normal young men was not influenced by prolonged wakefulness (112 hours). 2. Amytal given during such an experiment slightly depressed the output of these substances.

[8] Pincus, G., Hadidian, Z., and Yeaton, M., *Federation Proc.*, 1946, **5**, 81.

15365

Subtilin—Antibiotic Produced by *Bacillus subtilis*.* III. Effect on Type III Pneumococcus in Mice.†

A. J. SALLE AND GREGORY J. JANN.

From the Department of Bacteriology, University of California, Los Angeles.

In a previous communication[1] subtilin was found to exert a pronounced antibiotic action against a number of types of the pneumococcus by the agar cup-plate procedure. This ac-

TABLE I.
Treatment of Pneumococcus Type III Pneumonia in Mice.

		3 mice used in each group		
Date	Time	Controls	Treated immediately	Treated after 1 hr
2/14/46	12:30 p.m.	Infected	Infected	Infected
”	12:30 ”	—	0.5 cc subtilin	—
”	1:30 ”	—	”	0.5 cc subtilin
”	5:30 ”	—	”	”
”	9:30 ”	—	”	”
2/15	8:00 a.m.	First dead	—	—
”	9:30 ”	—	0.5 cc ”	0.5 cc ”
”	12:30 pm	Second ”	”	”
”	4:00 ”	Third ”	—	—
”	5:30 ”	—	0.5 cc ”	0.5 cc ”
2/16	9:30 a.m.		0.5 cc ”	0.5 cc ”
3/2	Results	All mice dead	All mice living, apparently normal	All mice living, apparently normal

* This investigation was aided by a grant from Eli Lilly and Company, Indianapolis, Indiana.

† The subtilin preparation used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, Calif.

[1] Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 60.

TABLE II.
Treatment of Pneumococcus Type III Pneumonia in Mice.

Date	Time	Six mice used in each group					
		Controls	Treated immediately	Treated after 1 hr	Treated after 3 hr	Treated after 6 hr	Treated after 9 hr
2/19/46	9 a.m.	Infected	Infected	Infected	Infected	Infected	Infected
,,	9 ,,	—	0.5 cc subtilin	—	—	—	—
,,	10 ,,	—	—	0.5 cc subtilin	—	—	—
,,	12 m.	—	0.5 cc ,,	,,	0.5 cc subtilin	—	—
,,	3 p.m.	—	,,	,,	,,	0.5 cc subtilin	—
,,	6 ,,	—	,,	,,	,,	,,	0.5 cc subtilin
,,	9 ,,	—	,,	,,	,,	,,	,,
2/20	9 a.m.	1st dead	,,	,,	,,	,,	,,
,,	12 m.	2nd and 3rd dead	,,	,,	,,	,,	,,
,,	3 p.m.	—	,,	,,	,,	,,	,,
,,	6 ,,	4th dead	,,	,,	,,	,,	,,
,,	9 ,,	5th and 6th dead	,,	,,	,,	,,	,,
2/21	5 a.m.	—	,,	,,	,,	,,	,,
3/7	Results	All dead	All living	All living	All living	All living	All living

tion was shown to be bacteriostatic in high dilution and bactericidal in more concentrated solution.

In the present communication subtilin was tested for its effect upon Type III pneumococcus *in vivo*.

Experimental. Nine white mice, weighing between 20 and 25 g, were injected intraperitoneally with 0.1 cc of a 24-hour serum broth culture of *Diplococcus pneumoniae*, Type III. Three of the mice were not treated but served as controls; 3 were treated immediately; the remaining 3 were treated one hour later. Each mouse was injected intraperitoneally with 0.5 cc of a solution containing 0.1 mg subtilin per cc (½ unit)[2] and every 4 hours thereafter throughout the first day. Three injections were given on the second day and one on the third. The schedule of treatments and results obtained are recorded in Table I. It may be seen that all of the control mice died in about 24 hours. On the other hand, all of the treated animals were living 2 weeks after treatment was discontinued. The animals were discarded after this period of time.

In a second series a larger number of animals (36) was used to check the results obtained in the first experiment. Each mouse was injected intraperitoneally with 0.1 cc of a 24-hour serum broth culture of Type III pneumococcus. The treated animals were each given 0.5 cc of a solution containing 0.1 mg subtilin per cc. Six mice were not treated but used as controls; 6 were treated immediately; 6 were treated after an interval of 1 hour; 6 were treated after 3 hours; 6 after 6 hours; and 6 after 9 hours. The animals were given another treatment 3 hours after the first injection, and every 3 hours thereafter until 9 p.m., then continued the following morning. The treatments were continued for approximately 48 hours. The results are recorded in Table II. It may be seen that all of the control mice died in from 24 to 36 hours after being given an injection of Type III pneumococcus. On the other hand all of the treated mice survived after being given subtilin for only 48 hours.

[2] Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 23.

The 6 mice in the last group (9-hour interval) were in very bad condition before treatment was started. After the second or third injection they appeared almost normal and after 24 hours they appeared to be free from any symptoms of pneumonia. All of the mice were observed for an additional 14 days without any treatment, then discarded. The antibiotic did not produce any observable toxic reaction in the mice.

Conclusions. Subtilin has been shown to exert a powerful *in vivo* action on the course of experimental pneumococcus Type III infections in mice. Animals treated with subtilin 9 hours after being injected with the organism were quickly cured of the infection. The antibiotic exhibited no apparent toxic reaction in the animals.

15366

Effect of Anterior Pituitary Growth Hormone on Urinary Nitrogen Loss Following Fracture.*

LESLIE L. BENNETT, ADRIENNE P. APPLEGARTH, AND CHOH HAO LI.
From the Institute of Experimental Biology and the Division of Physiology, University of California, Berkeley.

Introduction. It was noted by Cuthbertson[1] that bone fracture in man produced a rise in urinary nitrogen excretion, which, at its height, was not affected by a high protein diet. It was shown that this was due to more than disuse atrophy, as splinting of the limbs in healthy individuals produced only a slight rise. Howard and co-workers[2] have confirmed Cuthbertson's work in man, and have demonstrated that there was a long period (average 35.6 days) during which patients were in actual negative nitrogen balance. In a subsequent publication[3] these same workers found no beneficial effect from increased protein in the diet, the added protein being excreted quantitatively in the urine.

In rats, Cuthbertson[4] noticed a similar rise following fracture. The maximum nitrogen excretion occurred on the third day, and the animals had returned to normal after 5 days. Cuthbertson also found that the amount of nitrogen excreted was not accounted for by the loss of weight in the limb, but rather that tissue breakdown seemed to be occurring generally throughout the body. Cuthbertson, Webster and Young[5] found that a crude alkaline extract of the anterior pituitary prevented the rise in nitrogen excretion. In view of these findings it seemed of interest to determine whether any effect could be obtained with growth hormone, inasmuch as it would be expected to cause nitrogen retention.

Methods. Rats of the Long-Evans strain approximately 50 days old were used. In order to ensure a constant food intake during the whole experimental period, all animals were restricted to 10 g of stock diet per day. Twenty-four males were studied and were divided into 4 groups of 6 each. The first group was retained as a normal control, the second unoperated but given growth hormone, the third operated, and the fourth operated and given growth hormone. The growth hormone was a homogeneous preparation and was isolated by the procedure previously de-

* Aided by grants from the Research Board of the University of California and the Rockefeller Foundation, New York City.

1 Cuthbertson, D. P., *Lancet*, 1942, **1**, 433.

2 Howard, J. E., Parson, W., Stein, K. E., Eisenberg, H., and Reidt, V., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 156.

3 Howard, J. E., Winternitz, J., Parson, W., Bigham, R. S., Jr., and Eisenberg, H., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 209.

4 Cuthbertson, D. P., McGirr, J. L., and Robertson, J. S. M., *Quart. J. Exp. Physiol.*, 1939, **29**, 13.

5 Cuthbertson, D. P., Webster, T. A., and Young, F. G., *J. Endocrin.*, 1941, **2**, 468.

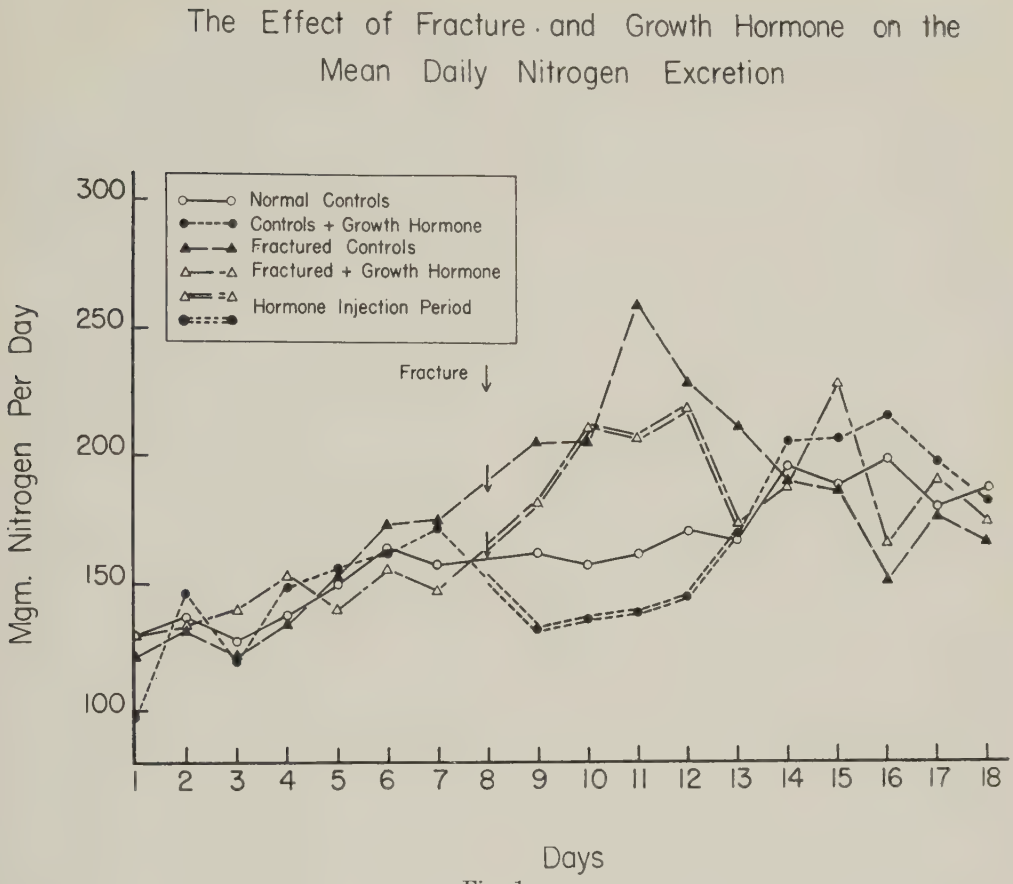

Fig. 1.

scribed.[6]

Following a preliminary period of adaptation to diet and cages, the nitrogen excretion was followed daily for a 7-day control period. At this time, both femurs were fractured under ether anesthesia. An incision was made on the lateral aspect of the thigh; the muscles were freed from the bone; and a comminuted fracture of approximately ½ cm in length was produced in the middle third of each femur. The incision was then sutured. There was little bleeding, and, although rigid asepsis was not observed, there was no evidence of infection. Daily intraperitoneal injections of 1 mg of growth hormone were begun on the day of fracture and were continued for 5 days. The daily nitrogen excretion was followed for 5 more days after growth hormone injections were stopped. Nitrogen was determined by the micro-Kjeldahl method.

Results. In Fig. 1 are shown the curves of mean daily nitrogen excretion for the 4 groups. The normal control group showed a slight but steady rise in nitrogen excretion over the entire period. In the control period before fracture, the lines representing the nitrogen excretion of the 4 groups intermingled, but at the 8th day they separated characteristically. That of the unoperated group given growth hormone dropped abruptly after the first injection and remained low during the injection period, but rose above that of the normal controls after growth hormone was stopped. In the fractured group, the excretion rose on the first day after fracture, reached a maximum on the third, and fell off in the following few days. In the last

[6] Li, Choh Hao, Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1945, **159**, 353.

group, fractured and growth hormone treated, the rise occurred but was less than that in the fractured untreated group.

The mean level of nitrogen excretion for each of the 4 groups during the control period was essentially the same, running from 142 mg to 144 mg of nitrogen per day with a standard deviation of from ± 3.5 to ± 5.1. In the 5-day postoperative period the average daily excretion of the control group rose to 165 ± 4.8† while that of the control group given hormone rose only to 145 ± 4.3. This represents a daily retention of 20 mg of nitrogen for the treated group as compared with the controls for the same period. The average daily excretion of the fractured group rose from 142 ± 5.1 to 223 ± 9.0, while that of the group fractured and given growth hormone rose only from 143 ± 3.5 to 199 ± 8.0, representing a retention over the untreated fractured group of 24 mg of nitrogen per day. The differences between the groups noted in this first 5-day postoperative period were all significant having p values of .05 and less.[7]

In the 5-day period after growth hormone was stopped, the nitrogen excretion of the control group averaged 30 mg per day higher than in the previous period, while that of the growth hormone treated group rose 58 mg. Also, the nitrogen excretion of the group fractured and given growth hormone was higher on the average than that of the fractured untreated group.

Discussion. In these experiments the retention of nitrogen following growth hormone was approximately the same in the fractured and unfractured rats when they were compared with their respective untreated controls. It would appear that the effect of growth hormone was to lower the base line of nitrogen excretion upon which the stress of fracture was superimposed. Cuthbertson, Webster and Young[5] reported that a crude extract of the anterior pituitary completely prevented the rise of nitrogen excretion after fracture, although it did not promote nitrogen storage beyond that occurring in the uninjected control period. There are several important differences between our experiments and theirs which could explain the different results obtained. In the present experiment 2 legs were fractured, creating a greater tendency to breakdown of protein than in the experiment of Cuthbertson, Webster and Young, where only one leg was broken. In addition younger animals were used in our experiments, which may have reduced the nitrogen retaining effect of the growth hormone.

Summary. The daily urinary nitrogen excretion was followed in growth hormone treated rats with bilateral femur fractures. The normal and fractured rats given growth hormone showed approximately the same decrease in nitrogen excretion when compared with their respective controls.

† Standard deviation of the mean.

7 Fisher, R. A., *Statistical Methods for Research Workers,* Oliver and Boyd, London, 1936.

15367

Measured Dose of Gamma Hexachlorocyclohexane (γ 666) Required to Kill Flies and Cockroaches, and a Comparison with DDT.

J. SAVIT, J. J. KOLLROS, AND J. M. TOBIAS. (Introduced by R. W. Gerard).

From the University of Chicago Toxicity Laboratory and Department of Physiology.*

Hexachlorocyclohexane† or "666" has been said to be more toxic than DDT‡ for certain insects[2,5,7,8,9,11] and mammals.[3] As was also true of DDT until recently,[13] however, most of the information is in terms of percent kill after exposure for a given time to a given environmental concentration of the

* This work was carried out under contract with the Medical Division of the Chemical Warfare Service.

agent. There are very few data available[4] on the actual measured dose (mg per kg) required to kill insects. Exposure data are invaluable from the point of view of field application,[6] but for studies on mechanism of action, it is essential to know the approximate dose which an insect must receive to produce death or some other specific physiological effect.[14,15] Therefore, measurements have been made of the LD_{50} (mg per kg required to kill 50%, determined graphically and by the method of Bliss[1]) of the active gamma isomer of hexachlorocyclohexane for flies *(Musca domestica* and *Calliphora spp.)* and the cockroach *(Periplaneta americana)*.

Methods. Since the gamma isomer of hexachlorocyclohexane is far more toxic than the alpha, beta or delta forms, and is the important constituent of manufactured 666, it has been used in pure form (m.p. 112.5°C)[12] throughout, in these studies. All roaches have been unanesthetized, but flies were lightly anesthetized with ether during the administration of the toxic agent.

Methods of administration have been essentially the same as those used in a similar study of DDT.[13] The toxic agent, dissolved in acetone, was delivered through a 27-gauge hypodermic needle, from a 0.25 cc tuberculin syringe fitted with a micrometer screw-driven piston.[10] Such an instrument is capable of delivering volumes of liquid of the order of 0.2 mm^3 with about 5% accuracy. For injection purposes, in the roach, the needle was made to enter the abdomen beneath a sternite and near its hinge-like point of attachment. Upon withdrawal of a needle so inserted the sternite falls back into place and seals the hole. No leakage has been seen following such a procedure. For surface application, roaches were held by the wings. The γ 666 was then deposited beneath the wings on the dorsum of the thorax from the instrument described above, but now through a blunted needle held flat against the body surface. When folded back into place the seldom-moved wings protect against loss of dry powder remaining after solvent evaporation. Control animals were given acetone without the γ 666 (Tables I and II).

Roaches were kept in large battery jars, in groups of 10 or fewer, with food and water continuously available. Flies were kept in similar groups in 150 cc wide-mouthed bottles.

Results. The detailed data are shown in Tables I and II, and are summarized and compared with similar data for DDT in Table III. It will be seen (Tables I and II) that in the region of the LD_{50}, changes in dose of γ 666 made relatively little difference in terms of mortality. This plateau-like region of the toxicity curve is thought to be due to gross inhomogeneity of the test animals. Neither sexes nor specific ages were carefully selected and random variations in

† 1, 2, 3, 4, 5, 6, hexachlorocyclohexane or "666" is a mixture of alpha, beta, gamma and delta isomers. The name "Gammexane" refers to the active gamma isomer as does "γ 666."

‡ 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane.

1 Bliss, C. L., *Ann. Appl. Biol.*, 1935, **22**, 134.

2 Bracey, P., and David, W. A. L., (British) *Insecticide Development Panel*, 1944, (44), 170.

3 Cameron, G. R., and Burgess, F., (British) *Insecticide Development Panel*, 1944, (44), 131.

4 David, W. A. L., (British) *Insecticide Development Panel*, 1945, (45), 236.

5 Gersdorff, W. A., and McGovran, E. R., *Soap and Sanitary Chemicals*, 1945, **21**, 117.

6 Jenkins, D. W., U. S. Army Service Forces, Chemical Warfare Service, Medical Division Report No. 56, Edgewood Arsenal, Md., Sept. 26, 1945.

7 Kearns, C. W., Ingle, L., and Metcalf, R. L., *J. Econ. Ent.*, 1945, **38**, 661.

8 Madden, A. H., Lindquist, A. W., and Jones, H. A., *U. S. Dept. of Agric. Bur. Ent. and Plant Quar. Int. Rep.*, No. 0-101.

9 McGovran, E. R., Gersdorff, W. A., Fales, J. H., and Piquett, P. G., (British) *Insecticide Development Panel*, 1944, (44), 209.

10 McMaster, P. D., Rockefeller Inst. for Med. Research, N. Y. C., personal communication.

11 Richards, A. G., and Cutkomp, L. K., *Biol. Bull.*, in press.

12 Slade, R., (British) *The Hurter Memorial Lecture, Insecticide Development Panel*, 1945, (45), 237.

13 Tobias, J. M., Kollros, J. J., and Savit, J., *J. Pharm. and Exp. Ther.*, 1946, **86**, 287.

14 Tobias, J. M., and Kollros, J. J., in press.

15 Tobias, J. M., Kollros, J. J., and Savit, J., in press.

TABLE I.
Toxicity of γ 666 for the Cockroach *(Periplaneta americana)*.

Route	No. roaches	Vol. given mm³	γ 666 mg per kg	% mortality at hours indicated 24	48	72	96	120
Surface	10	1.0	0	0	0	0	0	0
	20	1.0	1	10	10	15	20	20
	22	0.4-1.0	4	36	45	50	50	50
	25	0.6-1.0	6	28	52	56	56	56
	10	0.8	8	60	70	80	80	80
	49	1.0	10	18	45	49	57	59
	20	1.0-2.0	20	20	60	65	70	75
Injection	10	1.0	0	0	0	0	0	0
	10	1.0	1	0	10	10	20	20
	10	1.0	4	20	50	60	70	70
	10	1.0	6	20	20	30	30	40
	20	1.0	10	20	65	75	80	80
	10	1.0	20	30	60	70	80	80

Graphically determined
Approximate 120-hr LD_{50} (surface), 5 mg per kg (range 4 to 7.5).
Approximate 120-hr LD_{50} (injection), 4 mg per kg (range 3 to 7.5).
By method of Bliss[1]
120-hr LD_{50} (surface), 4.57 mg per kg (fiducial limits 1.0-9.4 mg per kg).
120-hr LD_{50} (injection), 3.39 mg per kg (fiducial limits less than 0.5-1.3 mg per kg).

TABLE II.
Toxicity of γ 666 Applied to the Body Surface of Flies (in Acetone).

Species	No. flies	Vol. applied mm³	γ 666 mg per kg	% mortality at hours indicated 24	48
Musca domestica	25	0.4	0.3	16	20
(newly emerged, 1-18 hr)	25	0.6	0.4	52	60
	25	0.8	0.5	56	60
	22	1.0	0.7	91	95
(older adults)	31	1.0	0	3	3
	20	1.0	0.3	5	5
	30	0.4	0.5	30	30
	30	0.6	0.8	33	53
	50	0.6	1.4	64	67
	15	1.0	1.8	87	100
	10	0.4	2.7	90	90
	5	1.0	3.5	100	—
	20	0.6	6.0	100	—
	19	1.0	7.0	100	—
Calliphora spp.	33	0.4	0.5	24	42
(older adults)	20	0.6	0.7	10	60
	32	0.8	0.9	41	65
	15	1.0	1.2	80	100

Graphically determined
Approximate 48-hr LD_{50} (*Musca*-newly emerged), 0.4 mg per kg (range 0.4 to 0.5).
Approximate 48-hr LD_{50} (*Musca*-older adults), 1 mg per kg (range 0.7 to 1.2).
Approximate 48-hr LD_{50} (*Call. spp.*-older adults), 0.6 mg per kg (range 0.6 to 0.7).
By method of Bliss
48-hr LD_{50} (*Musca*-newly emerged), 0.4 mg per kg (fiducial limits less than 0.1-0.65 mg per kg).
48-hr LD_{50} (*Musca*-older adults), 0.83 mg per kg (fiducial limits, 0.63-1.1 mg per kg).
48-hr LD_{50} (*Call. spp.*-older adults), 0.6 mg per kg (fiducial limits, less than 0.1 to more than 1.2 mg per kg).

TABLE III.
Comparative Toxicity of γ 666 and DDT for Flies and Cockroaches.*

Insect	Approximate LD_{50}, mg per kg†			
	Surface		Injection	
	γ 666	DDT	γ 666	DDT
Periplaneta americana	5	10	4	5-8‡
Musca domestica				
(newly emerged)	0.4	2	—	—
(older adults)	1	8-21	—	—
Calliphora spp.				
(older adults)	0.6	9-28	—	—

* DDT data.[13]
† Roach observation period 120 hours, fly 48. Solvent acetone in all cases.
‡ Probably closer to 8.[13]
N.B. All data in this table graphically determined.

either or both of these categories could account for such a spread.

From Tables I and III it can be seen that the LD_{50} for γ 666 applied to the surface of the cockroach (4.6 mg per kg) is not significantly different from that for intra-abdominal injection (3.4 mg per kg). This approximate equivalence in toxicity of surface-applied and of injected material is a most interesting phenomenon, and has also been demonstrated, in the case of the cockroach, for DDT.[13] It is not known to the authors to occur with any toxic agent administered to mammals. The finding again highlights the importance of the extremely efficient absorption of certain substances by the insect body surface. It is most unlikely that this absorption took place through spiracles, since in the roach, where application was easily localizable, the toxic agent was placed on the body at a considerable distance from the spiracles, and inspection revealed rapid evaporation of solvent with little flow. In the case of DDT, in addition, there has been shown to be a positive correlation between the presence of a chitinous exoskeleton and susceptibility to the external application of the toxic agent.[11]

Gamma 666 is only about twice as toxic as DDT for the cockroach (Table III). For the adult fly, however, it is distinctly more toxic (LD_{50}, 0.8 mg per kg) than DDT (LD_{50}, 8 to 28 mg per kg). The data show that it is also somewhat more toxic ($p = 0.05$) for the newly emerged fly *(Musca domestica)* (LD_{50}, 0.4 mg per kg) than for the adult (LD_{50}, 0.8 mg per kg), but the decrease in sensitivity as the fly ages is less than that reported for DDT (Table III).[13] The toxicity data reported here suggest a somewhat greater absolute toxicity of γ 666 for adult flies than do those of David[4] who reported a maximum median lethal dose of 2 and 3 mg per kg for the male and female respectively of *Musca domestica.* The difference is small, however, and may not be a meaningful one.

Those flies *(Musca domestica)* and roaches *(Periplaneta americana)* which die after a surface applied LD_{50} dose of γ 666 do so more quickly than after a comparable dose of DDT.[13] In addition, although the times have not been measured, it is the strong impression of the authors that these insects show symptoms of poisoning (tremors, ataxia, convulsions, falling, prostration) much sooner after γ 666 than after a comparably toxic dose of DDT. This is important, since a faster "knockdown rate" implies less time for ranging and oviposition.

Conclusions. 1. The approximate LD_{50} for γ 666 in the cockroach *(Periplaneta americana)* is 4.6 mg per kg when applied to the body surface and 3.4 mg per kg when injected intra-abdominally. These values are not significantly different.

2. An approximate equivalence, similar to that noted above, between surface and injection toxicity for the roach has been reported for DDT. This finding emphasizes the importance of the absorptive capacity of the insect body surface for contact poisons

in contributing to the effectiveness of insecticides.

3. The approximate LD_{50} for γ 666 applied to the body surface is 0.4 mg per kg for the newly emerged fly *(Musca domestica)*. For the older adult the LD_{50} is about 0.8 mg per kg for *Musca domestica* and 0.6 mg per kg for *Calliphora spp.* This decrease in sensitivity as the fly ages is less than that reported for DDT.

4. Gamma 666 is about twice as toxic as DDT for the cockroach *(Periplaneta americana)*, and is distinctly more toxic than DDT for *Musca domestica* (newly emerged and adult) and *Calliphora spp.* (adult).

5. Both death and knockdown occur more rapidly after surface application of γ 666 to *Musca domestica* and *Periplaneta americana* than after DDT.

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Type-specific Capsular Swelling of Meningococci by Chicken Antiserum.

KELSEY C. MILNER AND MORRIS F. SHAFFER.

From the Department of Pathology and Bacteriology, School of Medicine, Tulane University, New Orleans, La.

Branham[1] has recently emphasized the value of serologic typing of meningococci for epidemiologic studies as well as for more exact etiologic diagnosis. The methods currently employed for this purpose include (a) capsular swelling with hyperimmune rabbit serum and (b) agglutination with monovalent antisera prepared in rabbits or chickens. One of the advantages of using chickens as a source of agglutinating serum is their ability to tolerate doses of meningococci which may be toxic or even lethal for rabbits.[2,3]

During the past 18 months we have had occasion to prepare such antisera in adult chickens. The course of injections was based on the report of Phair, Smith and Root.[2] Doses of 2 to 4 billion living organisms, derived from casein-hydrolysate starch agar cultures[4] and suspended in physiological saline, were given intravenously at intervals of approximately one week. After 4 injections the animals were allowed a rest period of 7 to 16 days before being bled; in some instances this bleeding was followed after 7 to 9 days by a fifth injection and the animals were kept 1 to 4 weeks longer before final exsanguination. The serum collected from the successive bleedings of each chicken was pooled and stored at 2°C. Throughout the period of immunization the birds appeared healthy and showed no appreciable loss of weight.

In testing for agglutinins, meningococci from cultures grown 5 to 18 hours on casein-hydrolysate starch agar were suspended in saline to a concentration of 2 to 4 billion cocci per ml. Dilutions of antiserum were mixed with an equal volume (0.3 ml) of culture suspension in small tubes and these were agitated vigorously on a Kahn shaking machine for 10 to 20 minutes at room temperature before readings were made. Agglutination was readily visible with the naked eye. The titer of the serum was recorded as the highest dilution, after addition of antigen, in which clumping was marked. The results summarized in Table I show that following immunization of chickens with 16- to 18-hour cultures or with cultures only 4 hours old, we obtained satisfactory agglutinating sera versus strains of meningococci

[1] Branham, S. E., *Am. J. Pub. Health*, 1945, **35**, 232.

[2] Phair, J. J., Smith, D. G., and Root, C. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 72.

[3] Miller, C. P., *Yale J. Biol. and Med.*, 1944, **16**, 519.

[4] Mueller, J. H., and Hinton, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 330.

TABLE I.
Summary of Data on Immunization of Chickens with Meningococci.

Animals employed			Immunizing antigens			Type-specific serologic properties of antiserum	
No.	Breed	Sex	Serologic type	Strain	Age of culture (hr)	Capsular swelling	Agglutination titer
68	Rhode Island Red	♀	I	1027	16-18	+	120
1	Plymouth Rock	♂	I	1027	4	+	240
7	White Rock	,,	I	1027	4	+	120
66	Rhode Island Red	♀	II	963	16-18	0	80
49	,, ,, ,,	,,	II α	1054	16-18	+	40
3	Buff Orpington	♂	II α	1054	4	+	160
8	White Rock	,,	II α	1054	4	+	320

Types I, II and II alpha, as others have done.[2,3,5]

Investigation was then made to determine whether these agglutinating sera would also exhibit the property of capsular swelling as is the case with rabbit anti-meningococcal sera of adequate potency. Suspensions of living organisms from 4- or 5-hour cultures on Mueller-Hinton medium were mixed in the usual fashion with a loopful of chicken antiserum plus a loopful of methylene blue stain. Microscopic observation revealed prompt and strikingly clear-cut capsular swelling of meningococci Types I and II alpha in the presence of their homologous antisera; no capsular swelling was demonstrable when cocci of these serologic types were mixed with any heterologous antiserum. No capsular swelling was seen in the mixtures of Type II meningococci with the single homologous serum available or with the several antisera versus the other 2 types. The above findings have been checked and found to hold true not only for the various sera as freshly drawn but also after storage at 2°C for periods from 8 to 14 months. The data in Table I also indicate that sex or breed does not markedly affect the success of immunization providing healthy adult birds, weighing 5 to 7 pounds, are employed.

Discussion. So far as we are aware, data on the use of chickens for the preparation of specific capsular swelling antisera against meningococci or other bacterial species have not been previously reported. The present findings are of interest in demonstrating that capsular swelling may be produced with antisera derived from animals other than the rabbit and the horse; they also suggest that chickens can be used to prepare quellung sera for bacteria to which the rabbit or horse may not respond favorably.

From a practical standpoint, the ease with which adult chickens can be successfully immunized for the production of quellung antiserum versus meningococci Types I and II alpha should make it feasible for many diagnostic laboratories to prepare a supply. Such sera can undoubtedly be used for rapid and direct serologic identification of meningococci in the spinal fluid as well as in cultures obtained from other sources.

5 Kabat, E. A., Miller, C. P., Kaiser, H., and Foster, A. Z., *J. Exp. Med.*, 1945, **81**, 1.

15369

The Activity of Anionic Surface Active Compounds in Producing Vascular Obliteration.

L. REINER.
From the Research Department, Wallace and Tiernan Products, Inc., Belleville, N. J.

The great variety of substances used for obliteration of varicose veins have the common property of being destructive to intima of the vein.[1,2] Some of the substances are

used in a strongly hypertonic concentration (glucose, sodium chloride) and dehydrate through osmosis; others form a highly alkaline solution which is incompatible with cell life even for a relatively short period of time (sodium carbonate, soap solutions); still others contain substances which are poisonous to the protoplasma of cells in general (quinine hydrochloride, halogens, mercuric chloride). Differences in the manner in which the intima of the vein is destroyed are suggested by the differences in the rate of thrombus formation after injection. Mercuric chloride produces thrombus in 3 or more days,[3] whereas complete obliteration takes place within 24 hours after the injection of 20% sodium salicylate solutions.[4] Even more pronounced are the effects produced by soap solutions.[1] As this may be owing at least in part to the surface activity of the fatty acid anions and the anionic micelles formed in aqueous solutions of soaps, it was of interest to study the effectiveness of the synthetic anionic detergents in obliterating veins. The results of this study are reported in the following.

Methods. The marginal ear vein of rabbits and the veins of dogs have been used frequently to study the thrombogenic and sclerosing properties of various agents.[5,6,7]

In preliminary experiments using the marginal ear vein of rabbits it was found that it is difficult to regulate the rate of dilution of the sclerosing solution with blood, which obviously is one of the important factors determining the extent of thrombus formation. Because of the great number of collaterals, dilution of the agent after injection was rapid and high concentrations of the agent had to be used. These destroyed the delicate marginal ear vein and its collaterals rapidly and completely, permitting free diffusion of the agent and producing inflammatory reactions and necrosis of the surrounding tissue. Even if thrombus was produced without much tissue irritation, it was often extended to collaterals and its length could not easily be measured.

Much better results were obtained when the tail vein of the mouse was injected. Here, again, high concentrations of the agents, 2% of sodium ricinoleate or the equivalent in sclerosing strength of the anionic synthetic detergents, caused gangrene within 24 to 48 hours and the entire tail sloughed off within a few days. However, if the injection was carried out at a very slow rate, thrombus could be produced using lower concentrations of most of the agents without much tissue irritation.

In order to obtain well reproducible averages it was necessary to standardize the procedure as follows: mice weighing 20 to 25 g were fasted for 20 hours before injection. They were placed in a holder which permitted free handling of the tail. The veins were distended by dipping the tail for 1 minute into water which was kept at a temperature of 45°C. The solution was injected from a 0.25 cc Tuberculin Syringe No. LT ¼ with divisions of 0.01 cc through a 26-gauge needle having a short bevel. The needle was inserted at a distance of 50 mm from the base of the tail so that the tip of the needle was about 45 mm from the base of the tail. Five-hundredths cc was injected over a period of 10 seconds. Dilution of the solution with blood was prevented for 50 seconds by compressing the vein at the base of the tail and at the site of the injection. The length of the thrombus was measured with a ruler. In the dose range used, thrombus was usually formed within 24 hours. Delayed thrombus formation was observed occasionally with the lowest concentrations used. The optimum time for reading giving the maximum average length of thrombus was at about 48 to 96 hours, although the differences in results obtained when the reading was made at any time

1 Ochsner, A., and Mahorner, H., *Varicose Veins*, The C. V. Mosby Company, St. Louis, 1939.

2 McPheeters, H. O., and Anderson, J., *Injection Treatment of Varicose Veins and Hemorrhoids*, F. A. Davis Company, Philadelphia, 1943.

3 Régard, G. L., *Rev. med. de la Suisse Rom., Genevé*, 1925, **65**, 102.

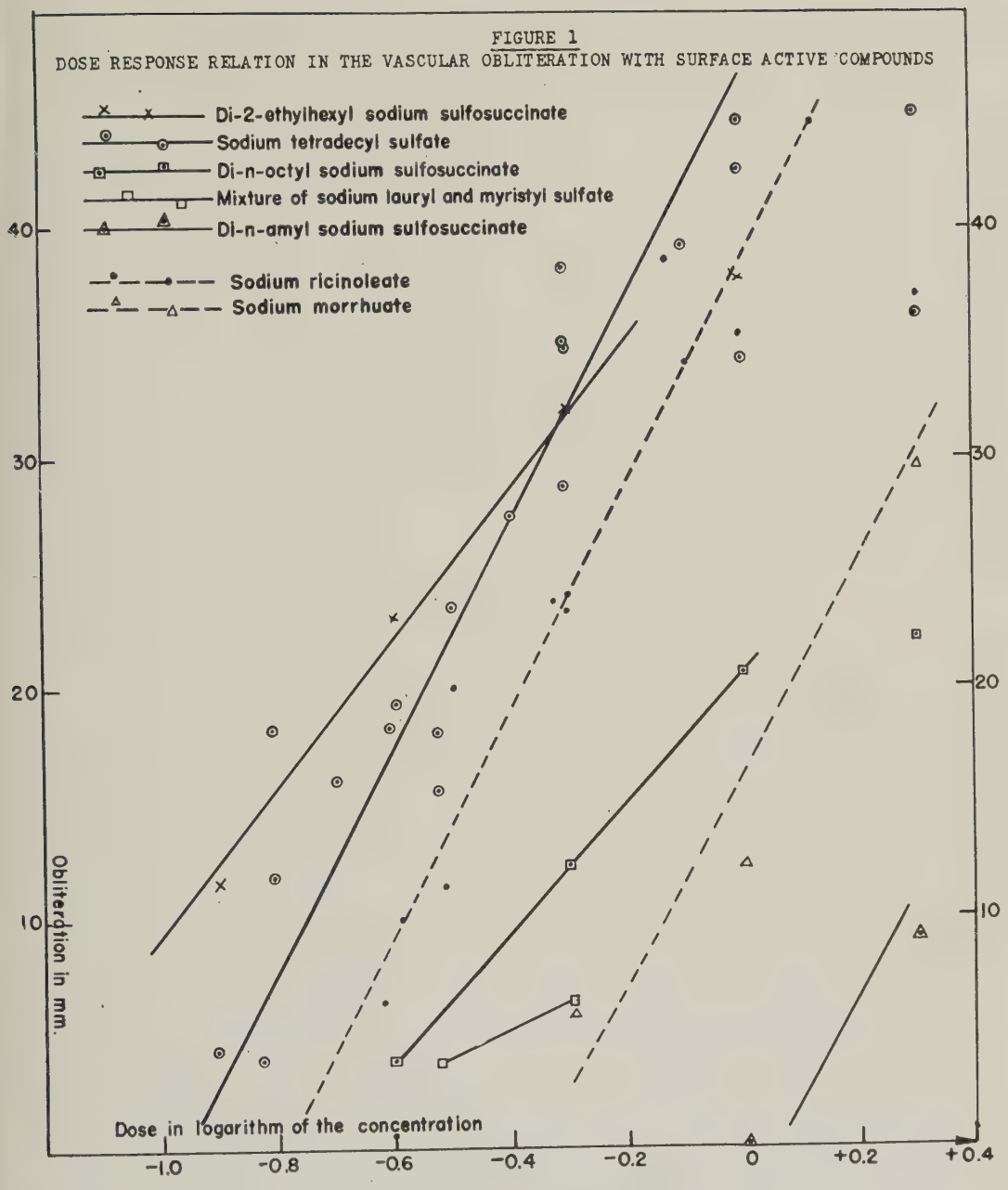
4 von Meisen, *Acta chir. Scandinav., Stockholm*, 1926, **60**, 435; 1927, **62**, 17.

5 Kilbourne, N. J., Dodson, V., and Zeiler, A. H., *Surg. Gynec. and Obst.*, 1932, **54**, 640.

6 Isaak, L., *Arch. Dermat. and Syphilol.*, 1940, **42**, 86.

7 Jensen, H., and Jannke, P., *J. Am. Pharm. A. (Scient. Ed.)*, 1944, **33**, 362.

FIGURE 1
DOSE RESPONSE RELATION IN THE VASCULAR OBLITERATION WITH SURFACE ACTIVE COMPOUNDS



between 2 to 7 days were not significant. The experimental points in Fig. 1 are averages of 2 to 3 readings made within 1 week after the injection. Each point represents the average length of the thrombus calculated from the results obtained with usually 10, but not less than 4 mice.

The average length of the thrombus decreased gradually during the latter part of the first week and subsequently. In the low-response range recanalization was frequent. In the high-response range permanent obliteration through organization took place. The extent of obliteration after resorption of the thrombus was measured in a few instances by dipping the tails in warm water, thereby

distending the patent veins, and it was found to be approximately proportional to the obliteration caused by thrombosis.

Materials Used. Extensive tests using 130 mice were made with a commercial sodium ricinoleate solution, pH 8.3, containing 5% of the soap and 2% of benzyl alcohol in water. Dilutions of 0.15 to 2.0% made with sterile distilled water were used.

The sodium morrhuate tested was also a commercial preparation containing 5% of the soap and 2% of benzyl alcohol in water. The solution was slightly turbid and had a pH of 10.1. Dilutions containing 2.0, 1.0, 0.5 and 0.25% of the soap were made with sterile distilled water. Ten mice were injected with each of these dilutions.

Sodium tetradecyl sulfate (sodium 2-methyl-7-ethyl-undecyl sulfate-4) was prepared by purification of the commercial product. It was a colorless, transparent, waxy solid which contained 90% of the detergent; the remaining 10% consisted of phosphate buffer and water. Water-clear solutions of pH 7.5 were made with water or 0.85% aqueous sodium chloride solution. They were sterilized by autoclaving for 20 minutes at 15 lb pressure. This procedure did not cause appreciable hydrolysis of the ester as the solutions remained water clear and their pH value remained the same or decreased only slightly. One hundred and seventy mice were used and the concentration of the solutions injected varied from 0.125 to 2%.

For testing the effectiveness of sodium dodecyl and sodium n-tetradecyl sulfate 0.5% and 0.3% solutions of Duponol were used. The pH of the solutions was 7.1. Ten animals were injected with each of the 2 solutions.

Four of the aerosol type of compounds, the di-2-ethylhexyl (aerosol OT) (I), di-n-octyl (II), diethylpropyl (III), and di-n-amyl (IV) sodium sulfosuccinates were obtained in a highly purified form from the American Cyanamid Company.* Clear, approximately neutral solutions in distilled water were prepared by warming, if necessary, and keeping at 40°C until injected. Ten mice were injected with each of the solutions containing 0.125, 0.25, 0.50 and 1.0% of I; 0.25, 0.50, 1.0 and 2.0% of II; and 1.0 and 2.0% of III and IV.

Results. In Fig. 1 the average length of thrombus is plotted against the logarithm of the concentration. It seems that a correlation exists between the concentration and the response, *i.e.,* the average length of the thrombus. With sodium tetradecyl sulfate and the soap solutions, the response was proportional to the logarithm of the concentration, *i.e.,* dose, up to a response of about 40 mm. The maximal individual response was about 45 mm as this was the length of the vein exposed to the drug. The slope of the rectilinear portion of the dose response curve was about the same for these substances. From the estimated position of the lines, the ratio of potencies, that is, the reciprocal of the ratio of the doses producing equal responses, was estimated to be 1.5 for sodium tetradecyl sulfate over sodium ricinoleate. The preparation of sodium morrhuate used in this study seemed to be considerably weaker in thrombogenic activity than either sodium tetradecyl sulfate or sodium ricinoleate.

The slope of the straight portion of the curve obtained with the 2 isomeric aerosols, I and II, appears to be smaller than the slopes obtained with the soap solutions or sodium tetradecyl sulfate solution, although the difference may not be significant. The branched isomer, I, was found to be a much stronger thrombogenic agent than the normal chain isomer, II. In low concentrations I was more active than sodium tetradecyl sulfate. The dipentyl sodium sulfosuccinates, III and IV, were much less active than the dioctyl compounds. Here the branched chain compound, IV, showed no activity in the concentration used, whereas the normal chain compound, III, showed a slight activity. The mixture of the normal chain sodium dodecyl and tetradecyl sulfate was much less active than the branched chain sodium tetradecyl sulfate.

Toxicity to Tissues. This was tested by injecting aqueous solutions of the compounds in concentrations given in Tables I and II in doses of 0.1 cc subcutaneously and in doses of 0.05 cc intradermally into rabbits. The

* Through the courtesy of Mr. Clyde Sluhan.

TABLE I.
Reactions Produced by the Subcutaneous Injection of Soap Solutions and Sodium Tetradecyl Sulfate. (Figures: diameters in mm; dose: 0.1 cc; reading: 48 hours after injection).

Concentration, %	Edema			Erythema		
	Sor	STS	Morrh	Sor	STS	Morrh
5	15	0	8	15	0	8
2.5	0	0	11	0	0	11
1.25	0	0	8	0	0	8
0.625	0	0	0	0	0	0

Sor: Sodium ricinoleate.
STS: Sodium tetradecyl sulfate.
Morrh: Sodium morrhuate.

TABLE II.
Reactions Produced by the Intradermal Injection of Soap Solutions and Sodium Tetradecyl Sulfate. (Figures: diameters in mm; dose: 0.05 cc; reading: 48 hours after injection).

Concentration %	Necrosis			Hemorrhage			Edema			Erythema		
	Sor	STS	Morrh	Sor	STS	Morrh	Sor	STS	Morrh	Sor	STS	Morrh
5	11	5	8	6	3	7	13	6	10	13	6	15
2.5	10	7	6	0	2	0	14	12	13	14	12	14
1.25	9	3	6	0	0	0	11	3	12	12	8	12
0.625	6	0	6	0	0	0	9	6	6	9	6	11
0.313	4	0	6	0	0	0	6	0	6	6	0	11

Sor: Sodium ricinoleate.
STS: Sodium tetradecyl sulfate.
Morrh: Sodium morrhuate.

results in Table I show that sodium tetradecyl sulfate produced no appreciable irritation upon subcutaneous injection and that it was definitely less irritating than the soap solutions. The intradermal injections did not show significant differences among these compounds although the edema and erythema produced by sodium tetradecyl sulfate appeared to be less than that produced by soaps.

Acute Toxicity. Purified sodium tetradecyl sulfate (Tergitol-4) was found by Smyth *et al.*,[8] to have a toxicity of $LD_{50} = 2$ gm/kg when tested on rats by feeding a 25% aqueous solution by stomach tube. In testing the toxicity of Aerosol OT in mice Lorenz *et al.*,[9] found that 1 mg but not 2.5 mg is tolerated when given intraperitoneally and that 1.25 mg was lethal to 1 out of 25 mice weighing 25-30 g. The oral LD_{50} of Aerosol OT in mice has been reported to be 1.5 g/kg.[10]

The intravenous route of administration was chosen for acute toxicity determinations as this is used for therapy with these substances. The mice were fasted for 24 hours and injections of 0.1 cc of various dilutions were given over a period of 20 seconds. Most deaths occurred within 24 hours and no change of survival rate was found after 1 week. LD_{50} was estimated graphically,[11] to be 90 ± 5 mg/kg for sodium tetradecyl sulfate using 47 mice, 100 ± 30 mg/kg for Aerosol OT using 15 mice, 100 ± 3 mg/kg for sodium ricinoleate using 24 mice, and 150 ± 45 mg/kg for sodium morrhuate using 35 mice. Thus only slight differences were found in the toxicity of these substances. The value obtained for Aerosol OT is in fair agreement with the results of Lorenz *et al.*,[9] ($LD_{50} > 40$ and < 100 mg/kg), in view of the fact that the rate of injection which was not specified by these authors might have been greater than that used in these experiments.

Chronic Toxicity. Smyth *et al.*,[8] reported that sodium tetradecyl sulfate did not pro-

[8] Smyth, H. F., Seaton, J., and Fischer, L., *J. Indust. Hygiene and Toxicol.*, 1941, **23**, 478.

[9] Lorenz, E., Shimkin, M. B., and Stewart, H. L., *J. National Cancer Institute*, 1940, **1**, 353.

[10] Benaglia, A. E., Robinson, E. J., Utley, E., and Cleverdon, M. A., *J. Indust. Hygiene and Toxicol.*, 1943, **25**, 175.

[11] Miller, L. D., and Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 261.

duce appreciable toxicity when approximately 150 mg/kg were taken daily in the drinking water by rats for 30 days. Benaglia *et al.*,[10] gave doses varying from 0.19 to 0.87 g/kg to rats with the food and doses between 0.1 and 0.5 g/kg to rabbits, monkeys, and dogs for 24 hours without finding appreciable toxicity, although some inhibitory effect on the growth rate was discernible.

Repeated intravenous injection with these solutions could not be carried out. Among the practicable routes in mice, intraperitoneal injection most closely approximates the intravenous route. Hence, 0.1 mg sodium tetradecyl sulfate in 0.1 cc was injected into 19 mice first intravenously, then 12 times intraperitoneally in the course of 3 weeks. Four mice died of peritonitis or intercurrent infection during the first week. The average weight of the remaining mice increased from 25.9 g to 31.3 g. Five of the surviving mice showed localized infections (abscess in the spleen, localized peritonitis, and adhesions). These were obviously due to perforation at the time of injection. Sections made of the lungs, liver, kidney, spleen, brain, intestine, stomach, testis, and heart of the remaining 10 mice showed no pathology attributable to the drug.†

Summary. The thrombogenic activity of some soaps and synthetic detergents was compared by injecting their solutions under standardized conditions into the tail vein of mice. Sodium tetradecyl sulfate (2-methyl-7-ethyl-undecyl sulfate-4) and Aerosol OT (di-2-ethylhexyl sodium sulfosuccinate) were found to be more potent agents than the soap solutions generally used to sclerose veins. Alkyl sulfates and sulfonates containing large branched chain hydrophobic residues were found to produce thrombus more readily than their straight chain isomers. Sodium tetradecyl sulfate produced less tissue reaction than sodium ricinoleate or sodium morrhuate. Its toxicity was not significantly greater than that of the soap solutions studied.

† Thanks are due Dr. I. E. Gerber, pathologist, for the preparation and examination of the slides.

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Sulfonamide and Penicillin Resistance of Group A Hemolytic Streptococci.*

LOWELL A. RANTZ, ELIZABETH RANDALL, WESLEY W. SPINK, AND PAUL J. BOISVERT.

From the Laboratories, Department of Medicine, Stanford University School of Medicine, San Francisco, Calif.

It has frequently been possible to establish satisfactory prophylaxis against Group A hemolytic streptococcus respiratory infection and its complications by the daily administration of a sulfonamide.[1] Such a technic, established by the U. S. Navy in many activities early in December, 1943, was uniformly satisfactory[2] until mid-summer of 1944. At that time strains of hemolytic streptococci that were resistant to the antibacterial action of these agents appeared[3] as the cause of disease among personnel in a station in the northwest and initiated an epidemic in spite of the widespread use of chemoprophylaxis. Later these organisms were transmitted widely to other Navy activities through the nor-

* This study was carried out under the auspices of the Commission on Hemolytic Streptococcal Infections, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

1 U. S. Department of Labor, Children's Bureau Publication No. 308, Washington, 1945, 64-87.

2 NAVMED 284, Bureau of Medicine and Surgery, Navy Department, Washington, D. C., 1944.

3 a. Epidemiology Unit No. 22, *J. A. M. A.*, 1945, **129**, 921; b. Damrosch, D. S., *J. A. M. A.*, 1946, **130**, 124; c. Eckles, L. E., personal communication, Sept. 28, 1945.

mal movements of men in training and have also established themselves among Army Air Force personnel.[4] Strains of only 3 Lancefield types, in order of degree of sulfonamide resistance, 17, 19 and 3, have been isolated under the circumstances just described.[3,4] Similar properties have been discovered in a few strains of other types, including 6 and 14.

The development of epidemics of respiratory infection caused by sulfonamide resistant hemolytic streptococci has been explained by 2 quite different hypotheses. One suggests that the etiological agents possessed a natural ability to multiply in the presence of sulfonamides and were therefore able to initiate outbreaks of disease in a host population in which sulfonamide prophylaxis had been established. Alternatively, it is proposed that mutants of previously sensitive strains appeared during rapid epidemic transmission which were resistant to the effect of sulfonamides. Such mutations were enabled to survive and spread because their new property was advantageous in the chemically altered host environment.

It seemed possible that a study of the sulfonamide resistance of a large number of strains of hemolytic streptococci collected during a study carried out between December 12, 1943 and April 20, 1944 in 2 army posts might give information as to which of the above concepts is correct. There was little possibility that sulfonamide resistant streptococci could have developed in naval activities and been transferred in so short a time to these particular army installations, since both were situated in an area remote from any naval training station. The nature and personnel of Post I has been described elsewhere.[5] Post II was an Army Air Base located about 10 miles from Post I. Contact between the 2 was possible through the intermingling of troops in a neighboring community.

Methods. Hemolytic streptococci were isolated from the upper air passages of infected men by technics described elsewhere.[6] The organisms were divided into groups and types by the precipitin technics of Lancefield. Sulfonamide sensitivity was determined by the method of Wilson;[7] penicillin sensitivity by streaking the streptococci on segments of blood agar plates containing appropriate amounts of the drug.

Results. Sulfonamide Sensitivity. The Group A hemolytic streptococci available for study consisted of 77 single, and 271 paired cultures of the same type, isolated from the upper air passages of 348 infected men at Post I. Each pair was recovered from the same individual, separate isolations having been made in the various cases at intervals from 1 to 25 days. In addition, 41 strains obtained from as many cases at Post II were tested.

Identical values for sulfonamide sensitivity were obtained with 225, and one-tube differences with 46, pairs. There were two-tube differences in 4 and these pairs were excluded. There was found no relationship between the time of isolation of the culture or the therapeutic administration of a sulfonamide and the variations between members of the pairs. It seems proper to assume that each pair of cultures represents the same organism recovered on 2 different occasions. The results indicate that the method for determination of sulfonamide sensitivity is quite precise and that relatively small differences in resistance may be satisfactorily compared.

The results of the determination of sulfonamide sensitivity of the single strains and of the nonidentical pairs (considering each member of the pair as a single strain) are presented in the first 4 columns of Table I. The values obtained for the 225 identical pairs are recorded in the 5th to 8th columns. The whole is summarized in columns 9 to 13.

No strain grew in the presence of more than 5 mg of sulfadiazine. Those of 5 types, 19, 24, 26, 30, and 36, as well as those included with the miscellaneous types, occasionally were

[4] a. Mitchell, R. B., Van Ravenswaay, A. I., Special Report, Office of the Air Surgeon, Oct. 8, 1945; b. Connor, A. R., Special Report, Office of the Air Surgeon, Oct. 8, 1945.

[5] Rantz, L. A., Rantz, H. H., Boisvert, P. J., and Spink, W. W., *Arch. Int. Med.*, in press.

[6] Rantz, L. A., Boisvert, P. J., and Spink, W. W., *Arch. Int. Med.*, in press.

[7] Wilson, A. T., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 130.

TABLE I.
Ability of Strains of Group A Hemolytic Streptococci to Grow in the Presence of Sulfadiazine.

Type	Single strains and individual members of nonidentical pairs considered as single strains				Identical pairs (2 cultures of same type from same patient)				All strains (considering identical pairs as single strains)				
	No. of strains	Grew in control only	Grew in 1 mg	Grew in 5 mg	No. of pairs	Grew in control only	Grew in 1 mg	Grew in 5 mg	Total strains	Grew in 1 but not in 5 mg	Grew in 5 mg	% grew in 1 mg or more	% grew in 5 mg
1	14	3	11	0	12	3	7	2	26	18	2	76.9	7.5
3	23	7	9	7	17	7	6	4	40	15	11	65.0	27.5
6	9	2	5	2	3	0	3	0	12	8	2	66.6	16.7
17	16	1	5	10	20	0	3	17	36	8	27	97.5	75.1
19	21	12	9	0	38	30	8	0	59	17	0	28.8	0.0
24	2	2	0	0	8	7	1	0	10	1	0	10.0	0.0
26	5	3	2	0	15	15	0	0	20	2	0	10.0	0.0
30	10	6	4	0	19	19	0	0	29	4	0	13.8	0.0
36	24	13	9	2	33	33	0	0	57	9	2	19.3	3.5
44	5	4	1	0	6	3	1	2	11	2	2	36.4	18.2
46	8	0	4	4	12	2	7	3	20	11	7	90.0	35.0
Other types	32	17	13	2	42	30	11	1	74	24	3	36.5	4.0

resistant to the action of 1 mg per 100 ml of sulfadiazine but all but 5 strains failed to multiply in 5 mg per 100 ml of the drug. Strains of types 1, 3, 44, and 46 were often resistant to the action of 1 mg per 100 ml and, frequently, to that of 5 mg per 100 ml of sulfadiazine. Type 6 is represented by very few examples but nearly all were slightly sulfonamide resistant. The strains of type 17 were the least sensitive in that all but one grew in the presence of 1 mg per 100 ml and 27 of 36 in the presence of 5 mg of sulfadiazine per 100 ml.

Five strains of type 3 were collected at Post II; 4 grew in the control tube only, one in the 5 mg tube. Seventeen strains of type 19 were studied, only one of which grew in the 1 mg tube and none in the higher levels of sulfonamide. Seventeen of 19 strains of type 17 grew in the 1 mg and 4 in the 5 mg tubes.

The month of isolation of the resistant strains of types 3 and 17 was determined. All strains of type 3, able to grow in the presence of 5 mg of sulfadiazine, were isolated after March 1, 1944, since this type did not appear as a cause of disease in the post before this date. The results with type 17 were different in that 2 resistant strains were recovered in January and 6 in February of 1944.

Penicillin Sensitivity. The ability of all of the above listed strains to grow in the presence of penicillin was studied. Regarding them as individual strains, 54 were resistant to the action of 0.02, and 7 to that of 0.05 units per ml. All were inhibited by 0.1 unit per ml. If the paired strains only are considered, it is discovered that in only 5 of the 271 instances were both members of the pair able to grow in the presence of as much as 0.02 units of penicillin per ml. No relationship between the serological types of these slightly penicillin resistant strains was discovered.

Comment. This study has demonstrated that certain types of Group A hemolytic streptococci were significantly more resistant to the antibacterial action of sulfonamides than were others collected during the same period. It is unlikely that these strains had been transmitted through a host population undergoing sulfonamide prophylaxis. They

must, therefore, be regarded as having been naturally resistant to the action of these chemicals.

Strains of 2 of these types, 3 and 17, later became established as the etiological agents of epidemic hemolytic streptococcal respiratory infection in military establishments in which sulfadiazine was being administered prophylactically. When studied under these circumstances the degree of resistance of strains of type 3 was approximately that discovered in this study but that of strains of type 17 was much greater.[3,4]

It is probable, therefore, that both of the hypotheses previously mentioned, explaining the appearance of epidemic, sulfonamide resistant, hemolytic streptococci, are correct. Certain strains of Group A hemolytic streptococci were present in the national military population at the time that mass chemoprophylaxis was instituted that possessed two advantageous biological properties: moderate sulfonamide resistance and a high degree of communicability. These characteristics permitted these strains to spread and cause disease after their introduction into a host population undergoing sulfonamide prophylaxis. In some instances (type 17) mutation occurred and much more sulfonamide resistant variants appeared; in others (type 3) the resistance of the strain did not become greatly enhanced.

This study has not revealed a precursor for the resistant strains of type 19 that also caused epidemics. It is quite possible, however, that naturally resistant variants of this type were present in other areas.

The failure of other moderately resistant strains discovered during this study to establish epidemics may have been due to a lack of certain properties which permit a high degree of communicability or to the fact that they were never introduced into a group in which sulfonamide prophylaxis was in use.

No significantly penicillin-resistant strains of hemolytic streptococci were discovered during this study.

Summary. Strains of certain types of Group A hemolytic streptococci were discovered to be naturally resistant to moderate amounts of sulfadiazine. It is suggested that such organisms originated the epidemics of streptococcal disease among troops receiving sulfonamide prophylaxis, later becoming more resistant by mutation. No strains significantly resistant to penicillin were discovered.

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Effect of Atropine, Testosterone and Pitressin on Experimental Myocardial Infarction.*

S. S. MINTZ AND B. KONDO.† (Introduced by L. N. Katz).

From the Cardiovascular Department, Research Institute, Michael Reese Hospital, Chicago.

Recently Mokotoff and Katz[1] have made use of an experimental approach to determine the effect of drugs upon infarct size following coronary ligation. It was shown by this means that papaverine had a definite effect in reducing infarct size, while aminophyllin parenterally had a smaller, but definite effect. It was felt that other drugs might profitably be evaluated by this method. In the present report data obtained with atropine, testosterone, and pitressin are presented.

Atropine was chosen since it has been claimed recently to have a clinically beneficial effect in myocardial infarction.[2] This beneficial effect was attributed to an abolition of supposed vagus coronary constrictor tone.

* Aided by the A. D. Nast Fund for Cardiovascular Research. The department is supported in part by the Michael Reese Research Foundation.

† Herbert G. Mayer Fellow.

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2 Leroy, G. V., Fenn, G. K., and Gilbert, N. C., *Am. Heart J.*, 1942, **23**, 637.

Previous work from this department,[3] however, has shown that the vagi actually are tonic coronary dilators. Testosterone was chosen because clinical benefit had been claimed from its use.[4,5] Pitressin was used because it had been shown to be a powerful coronary vasoconstrictor[6] and might therefore be expected to act detrimentally on the course of infarct healing.

The procedure was essentially similar to that described previously.[1] In all, 47 dogs were used between controls (11), atropine-treated (13), testosterone-treated (11), and pitressin-treated (12) series. Using nembutal anesthesia (25 mg/kilo), the left anterior descending coronary artery and accompanying vein were tied completely in each dog. The animals were permitted to survive 6 weeks before being sacrificed for necropsy examination.

Atropine sulfate‡ was given subcutaneously in doses of 1.3 mg daily for 2 and 6 days per week for the remaining 4 weeks in all but 2 dogs. In these 2 dogs 0.65 mg of atropine was given twice daily for the entire 6-week period. Testosterone propionate§ was given subcutaneously in doses of 25 mg three times weekly for the 6-week period. Pitressin§ was given subcutaneously in doses of .20 pressor units daily for 2 weeks and 6 times weekly for the remaining 4 weeks.

At necropsy all hearts were opened, the ligation of the coronary artery was checked and the endocardial aspect of the infarct was traced on a glass plate. The area was determined by planimeter (*cf.* [1]). The coronary arteries were then injected and X-ray photographs were made in most instances as described previously.[1]

TABLE I.
Effect of Drugs on Infarct Size Calculated as $10^4 \times$ area of infarct (in cm^2)

heart weight (in g)				
	Control	Atropine	Testosterone	Pitressin
	639	447	1004	1140
	409	1118	763	619
	933	1026	683	872
	598	980	1815	1006
	922	1168	1075	762
	577	804	592	673
	1123	809	291	865
	1356	815	1099	742
	681	994	101	613
	1000	741	1555	596
	1049	612	102	506
		817		390
		690		
Arithmetic mean	844	851	826	734
Standard deviation	275	186	528	212

The data are summarized in Table I. It will be seen that no alteration in infarct size was produced by the 3 drugs used. The differences in values in each of the series and in their averages are statistically insignificant. It can therefore be concluded that within the experimental error of our method, atropine, testosterone, and pitressin in the manner administered had no demonstrable effect on the healing of experimentally produced myocardial infarcts.

The reported beneficial clinical effect of atropine and of testosterone therefore apparently operates in some way other than by coronary vasodilation. The failure of pitressin to increase infarct size outside the error of the method, suggests the possibility that its pressor effect in these dogs neutralized the coronary vasoconstriction.

It must be borne in mind that this experimental method is relatively crude and that finer differences may be concealed by inescapable variations in technic.

Summary. Atropine, testosterone, and pitressin failed to affect infarct size over a period of 6 weeks following experimental ligation of the left descending coronary artery.

We are indebted to other members of the department for their assistance and to Dr. L. N. Katz for suggesting the problem.

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‡ We are indebted to the Abbott Laboratories for the atropine.

§ We are indebted to Schering and Co. for the testosterone and to Parke-Davis and Co. for the pitressin.

15372

The Flow of Blood Supplying the Cardiac Atria.

JOHN R. SMITH AND IRA C. LAYTON.

From the Department of Internal Medicine and the Oscar Johnson Institute for Medical Research, Washington University School of Medicine, St. Louis, Mo.

Experimental evidence suggests that disturbance of the blood supply of the cardiac atria may lead to auricular fibrillation,[1,2] or to other auricular arrhythmias.[3,4] Aside from anatomical studies, little attention has been directed to the flow of blood to the atrial structures and to factors which may impair that blood supply. These experiments were undertaken in order to study the inflow of blood into one of the atrial arteries, and to note the effect of increased intraatrial tension upon the inflow.

Method. Dogs weighing about 15 kg were anesthetized with veterinary nembutal. In each experiment a Starling heart-lung preparation was set up. The use of the heart-lung preparation permitted closer observation and control of many features of cardiac performance. The largest, and most accessible atrial artery to perfuse was the *left anterior auricular artery*,[5] easily identified as a branch of the circumflex coronary artery at the base of the left auricular appendage. This artery was dissected free; the left circumflex artery was also dissected from its origin for a distance of about 2 cm. A flexible rubber tube, with small glass cannula, was led from the aortic cannula and was inserted into the left circumflex about 2 cm from its origin so as to perfuse the vessel distally. Such perfusion of the circumflex served to maintain myocardial function. The circumflex was ligated at its origin, and a second tube (of metal) was led from the aortic cannula, through the flow-meter, and attached to the proximal segment of the circumflex vessel (as shown in Fig. 1). Arteries arising from this segment, other than the auricular, were securely ligated.

Inflow of blood into the auricular artery was measured, phasically and quantitatively, by the differential pressure orifice-meter devised by Gregg and Green.[6] Calibration of the instrument and interpretation of the flow curves have been described by them, and these were carefully followed in the experiments. The flow curves were synchronized with the cardiac cycle by simultaneous recording either with an electrocardiograph, or with aortic pressure curves using a Wiggers manometer, on the same bromide paper.

Tension within the left auricle was measured by a simple manometer connected into one of the pulmonary veins close to the auricular wall. Estimates of cardiac output were made by collecting outflowing blood in a graduate or siphon-recorder. Twelve experiments were performed on 12 heart-lung preparations showing good function.

Results. For observing auricular arterial inflow under normal conditions, the output of the heart-lung was about 600 cc per minute (commensurate with the capacity of the heart). The blood pressure was adjusted to 100 mm Hg. Tension within the left atrium varied from 3-6 cm of water in different hearts. The venous reservoir was 12 cm above the right auricle.

The normal pattern of inflow into the auricular artery is shown in Fig. 2. The curve indicates that forward flows occur during ventricular systole and diastole. Abrupt interruptions of the stream occur at the onset of ventricular systole; early in ventricular diastole, there is momentary cessation of inflow followed by a short period of backflow.

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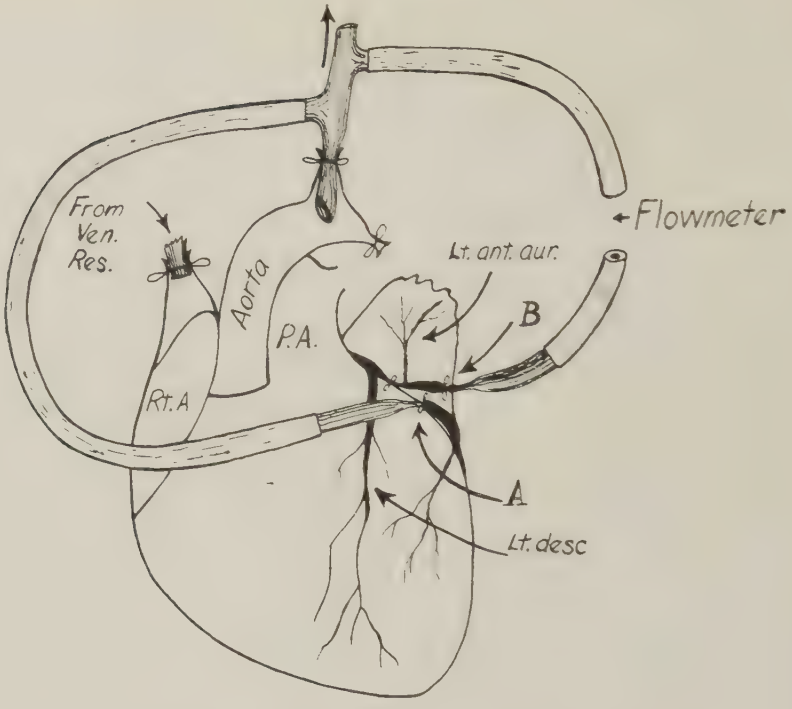

FIG. 1.

Diagram of the arrangement of cannulæ for the perfusion of the left anterior auricular artery. The left circumflex coronary artery is perfused distally (A) to maintain myocardial function. A second system, including the flowmeter, is used to perfuse a proximal segment of the left circumflex (B) of which the auricular artery is a branch.

The peak of rapid inflow in ventricular systole is slightly out of phase with the peak of aortic blood pressure owing, possibly, to delay in the stream flowing through the artificial circuit and flow-meter.

Quantitative analysis of the curves show that in most instances auricular arterial inflow during ventricular systole and diastole are nearly equal, systolic inflow being greater or lesser than that of diastole by a few hundredths cubic centimeter. In these hearts, total inflow for each cardiac cycle varied from 0.08 cc to 0.581 cc. A casual examination of the tracings suggests that the arterial stream during ventricular systole is of greater quantity than in diastole—a discrepancy which is more apparent than real. Rate of inflow in ventricular systole is greater for a short time, producing this form of curve. Tracings of blood inflow into the auricular artery resemble, in general, the metered curves of flow into the principal coronary arteries.[6]

Effect of Raising Left Intra-auricular Tension Upon Atrial Arterial Inflow: Elevation of intra-auricular tension was produced by: 1. induction of cardiac dilatation by raising cardiac output above optimum levels (4 experiments); 2. by inducing anoxia of the preparation (3 experiments), and 3. by producing cardiac dilatation by the administration of 20% chloral hydrate[7] (2 experiments). Cardiac dilatation by any of these methods readily caused a progressive rise of left intra-auricular pressure to 20-25 cm of water. During the period of myocardial dilatation, the blood pressure was maintained at 100 mm Hg. and the cardiac output showed only minor degrees of variation. Heart failure was not permitted to become more severe, for when

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TABLE I.
Examples of Quantity of Inflow into the Left Anterior Auricular Artery in 2 Normally Beating Heart-Lung Preparations.

	Cardiac output, cc per min	Blood pressure	Inflow systole, cc	Inflow diastole, cc	Total inflow, cc per cycle
Exp. 7	660	100	0.084	0.072	0.156
" 8	640	100	0.099	0.141	0.240

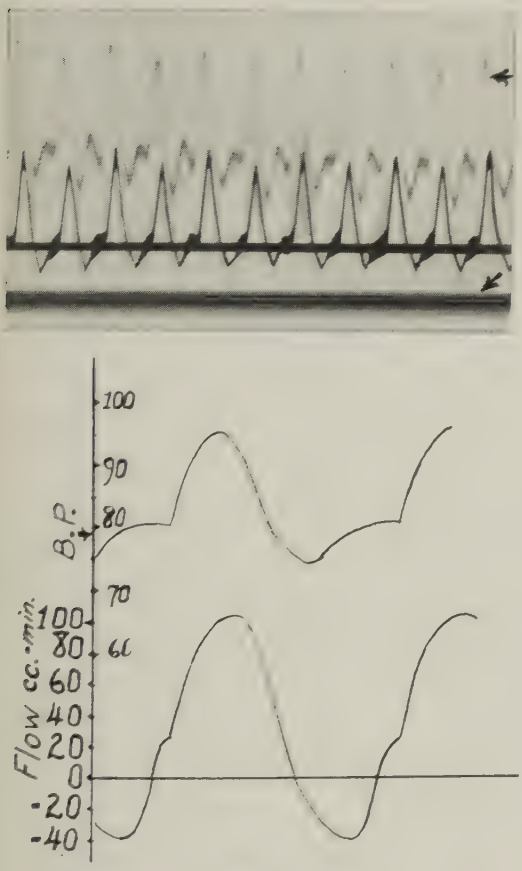

FIG. 2.

Upper: Segment of a curve showing aortic blood pressure tracing (marked by arrow), and the curve of blood inflow into the left anterior auricular artery in a heart-lung preparation. The flow curve has been inked to facilitate reproduction. Black line through the flow curve is line of zero flow; base line for blood pressure is marked by arrow. Lower: Blood pressure and inflow curves redrawn to linear ordinate scales. Analysis of flow curve shows inflow during ventricular systole of 0.249 cc; inflow during ventricular diastole of 0.332 cc. Inflow during cardiac cycle 0.581 cc. Cardiac output was 625 cc per minute.

marked failure occurred blood pressure and output could not be controlled, and the preparation became useless.

TABLE II.
Experiment 10, Normally Beating Heart-Lung Preparation, Blood Pressure Constant at 100 mm Hg. Quantitative Changes in Inflow into Left Auricular Artery During Rise of Left Intra-auricular Tension from Anoxemia.

Left intra-aur. tension, cm water	Cardiac output, cc per min	Aur. art. inflow per cycle, cc
3.3	580	0.140
8.8	640	0.123
24.0	600	0.096
8.8	610	0.136

With blood pressure and cardiac output remaining essentially constant, left auricular arterial inflow decreased as pressure within the auricle rose from myocardial dilatation. Decrease in the quantity flow became apparent as pressure within the auricle rose 8-10 cm of water; diminution of inflow became more marked as auricular tensions increased to 15-20 cm of water (Table II). In general, a rise of intra-auricular tension to 15-20 cm of water, or more, reduced arterial inflow approximately 30-40%. As the heart gradually recovered, the inflow again increased as intra-auricular pressure declined. Analysis of the curves further indicated that the inflow in ventricular systole and diastole was usually uniformly decreased as distension of the auricle occurred. Exceptionally, the inflow during ventricular diastole was greatly curtailed, so that forward flow into the auricular artery took place almost entirely during contraction of the ventricles.

Discussion. These experiments utilized a system conveying blood directly from the aortic cannula of the heart-lung preparation to the left anterior auricular artery. Therefore, the phasic changes observed in the inflow curves depended upon aortic pressure and upon alteration of factors affecting the auricular vascular bed. They were not due to changes of flow in the left circumflex artery.

The pattern of the inflow curves, in the normally beating preparation, suggests that the systolic rise of aortic pressure produces a rapid forward progression of blood in the auricular artery. During ventricular diastole, the flow is less rapid than in systole but is somewhat more sustained. Forward flow is momentarily interrupted at the onset of ventricular systole and diastole, coincident with rapid changes in aortic pressure. Generally there is a sharp backflow from the auricular artery during the rapid fall of aortic blood pressure, early in ventricular diastole.

The diminution of inflow into the auricular artery accompanying elevation of left intraauricular pressure appears to result from changes in the resistance in the auricular vascular bed, since cardiac output and blood pressure were maintained during the period of mild heart failure. It has been suggested that stretching of the cardiac walls may attenuate the intramural vessels, diminishing their capacity.[8] Spalteholz[9] and Unger[10] have shown that the greater part of the venous blood from the left auricle passes by vein to the coronary sinus, but that numerous Thebesian veins exist to account for the escape of a portion of venous blood directly into the auricular cavity. Therefore, a rise of intra-auricular tension may diminish venous outflow from the Thebesian vessels. More probably the distension of the atrial wall exerts pressure upon all of the intramural vessels preventing the free passage of blood through them.

The results of these experiments warrant the suggestion that distension of the auricles, (*e.g.*, resulting from heart failure or valvular disease) may curtail the blood supply to the auricles, favoring the development of auricular fibrillation or other auricular arrhythmias.[2]

Summary. The flow of blood into the left anterior auricular artery was measured phasically and quantitatively in the heart-lung preparation. The curves indicate that forward flow into the auricular artery occurs during ventricular systole and diastole, with abrupt, momentary interruptions of inflow at the onset of ventricular systole and diastole. Elevation of tension within the left auricle diminishes auricular arterial inflow; inflow again increases as pressure within the auricle is restored to normal levels. It is suggested that the interference with auricular blood supply, due to increased intra-auricular tension (as in heart failure) may enhance the establishment of aberrant auricular mechanisms.

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15373 P

Pyroninophilic Structures of Liver Cells in Carbon Tetrachloride Poisoning.

A. ROSIN AND L. DOLJANSKI. (Introduced by L. Halberstaedter).

From the Department of Experimental Pathology, The Hebrew University, (Cancer Laboratories), Jerusalem, Palestine.

The present report is concerned with changes in the pyroninophilic structures of the liver cell, as an early effect of carbon tetrachloride poisoning.

In the hepatic cells of various mammals and lower animals a peculiar type of granulation has repeatedly been observed and described.[1] These structures are characteristic in that they stain an intensive red with methyl green-pyronin (Pappenheimer's meth-

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od). They vary in size and shape and may have the form of spheres, plump rods or lumps. They are often accumulated in the immediate neighborhood of the nucleus, but occasionally are spread over the entire cytoplasm. There is a clear relationship between the pyroninophilic granule content of the liver cells and the nutritional state of the animal. In livers of fasting animals or in animals fed on carbohydrate or fat only the pyroninophilic structures disappear; in animals kept on a diet rich in proteins and products of protein cleavage they are especially numerous. The pyroninophilic granules can be digested by protein splitting ferments; they give positive Millon's, ninhydrin, diazo, and nitroprusside reactions. These facts led Berg to conclude that the pyroninophilic structures are paraplasmatic accumulations of proteins with a substantial proportion of lower degradation products. According to this author changes in the size and number of pyroninophilic granules are an indication of a disturbed protein metabolism.

The views of Berg were corroborated by a number of investigators.[2-9] Various authors[10-14] have opposed his opinions. Kremer[15] held the pyroninophilic structures to be the products of biliary secretion. The histochemical studies of Brachet[16] and of Biesele[17] made it probable that the structures observed by Berg contain ribonucleic acid as an essential component.

Few studies have been made on pyroninophilic structures under pathological conditions. It has been claimed[18] that in human livers showing cloudy swelling the pyroninophilic material is augmented. In livers of patients dying with severe infections, a marked increase of pyroninophilic granules could be observed.[19]

It has been further noted that administration of adrenalin as well as insulin considerably reduces the amount of pyroninophilic granulations.[3,5,7] Recently Korenchevsky[20] described a decrease in number and size of the pyroninophilic granules in gonadectomized rats, and a return to normal after injection of sex hormones.

In the present investigation we used young albino rats kept on a balanced diet, rich in proteins. Chemically pure carbon tetrachloride was given intraperitoneally in an amount of 0.1 ml per 100 g of body weight. The animals were sacrificed after various intervals. The liver samples were fixed in Zenker's and Carnoy's fluids immediately after removal and embedded in paraffin. The sections, 5 μ thick, were stained with methyl green-pyronin, according to Pappenheimer.

In the liver of normal uninjected rats fed on our standard diet, sufficient in every respect, pyroninophilic structures were invariably present in practically every hepatic cell. They were numerous, of varying size and shape and were fairly evenly distributed throughout the hepatic lobule. The aspect was quite different after administration of carbon tetrachloride. In the animals sacrificed one hour after injection of carbon tetrachloride the pyroninophilic granules in the periphery and the middle zone of the

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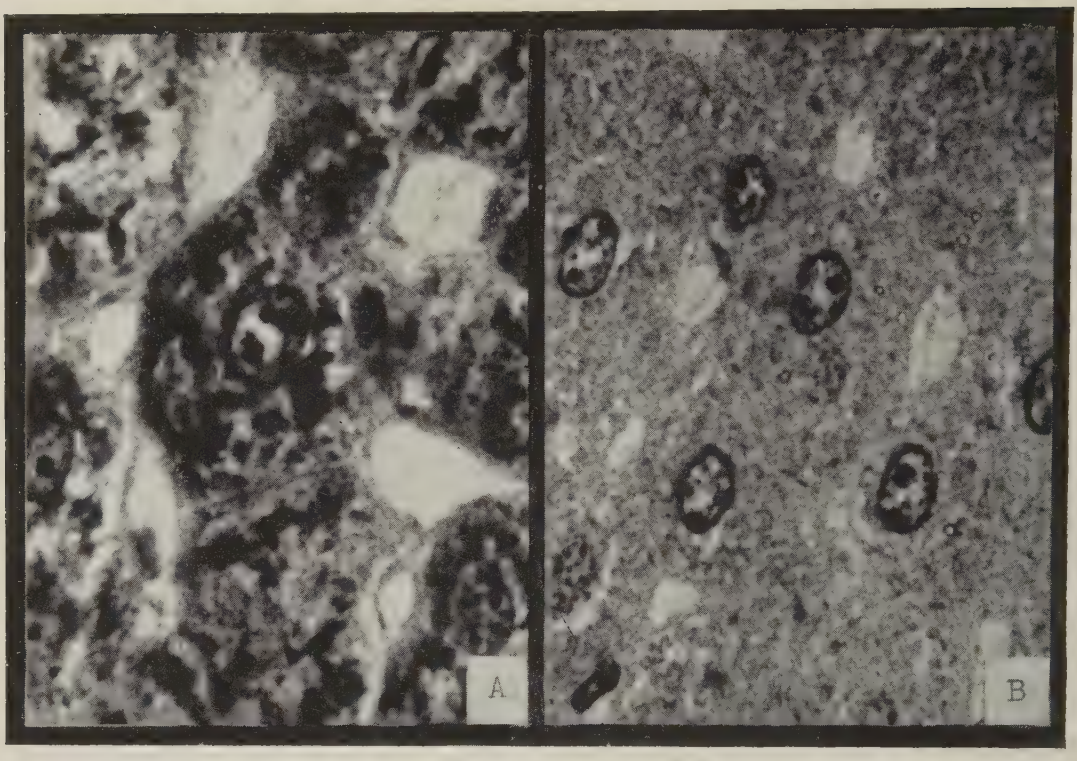

FIG. 1.
Liver of the rat (No. 293) sacrificed 1 hour after the injection of carbon tetrachloride. Fixed in Carnoy's fluid. Stained with methyl green-pyronin.
A. Liver cells from the periphery of the lobule. Mag. × 1900.
B. Liver cells from the central part of the lobule. Mag. × 1500.

lobule were essentially normal in size, distribution and number. In contrast, in the central zone of the lobule the parenchymal cells were free from pyroninophilic material and no traces of red granules could be identified. The line of demarcation between the empty central areas and those containing pyroninophilic granules is sharp. At this period no other noteworthy alterations were observed in the liver parenchym of the treated rats.

Summing up, the administration of carbon tetrachloride, as early as one hour after injection, brings about an alteration of the pyroninophilic structures, leading to their complete disappearance in the hepatic cells in the central part of the lobule. Inasmuch as the nature of the pyroninophilic structures is still a subject of discussion, no conclusive evidence pointing to the significance of these changes can be presented at time. If Berg's concept is correct, the early disappearance of the pyroninophilic granules in liver cells after injection of carbon tetrachloride can be regarded as a proof that disturbed protein metabolism in liver cells is one of the very earliest effects of carbon tetrachloride poisoning.

15374

Histamine Antagonists. V. Comparison of Benadryl and Pyribenzamine in Histamine and Anaphylactic Shock.

Sidney Friedlaender, Samuel M. Feinberg, and Alan R. Feinberg.
(Introduced by C. A. Dragstedt).

From the Division of Allergy, Department of Internal Medicine, Northwestern University Medical School, Chicago, Ill.

Following the lead of French investigators, several new antihistaminic compounds have recently been synthesized in this country and made available for experimental and clinical trial. B-dimethylaminoethyl benzhydryl ether (Benadryl) and pyridil-N′-benzyl-N-dimethylethylenediamine (Pyribenzamine), an analogue of the later French compounds, have proven effective in histamine,[1–3] and anaphylactic[3–5] shock and in the management of some allergic conditions in man.[6–10]

In order to have a basis for a comparative activity of these and similar compounds it was felt that an experimental study of these substances by the same technic and in the same laboratory was required. The present experiments deal with the comparative efficacy of Benadryl* and Pyribenzamine* in fatal histamine and anaphylactic shock in guinea pigs.

Histamine Shock. Adult male guinea pigs were given injections of histamine in the dorsal vein of the penis. Histamine phosphate was employed in increasing doses in a series of control animals to determine the 100% lethal dose. (All values of histamine are expressed in terms of the base). Another group of guinea pigs received 3 mg/kg of Benadryl intraperitoneally 15 minutes before the administration of histamine. A third group of animals was similarly prepared with 3 mg/kg of Pyribenzamine (Table I). In the untreated control group, 0.4 mg/kg of histamine resulted in the death of all animals within 5 minutes. At lower doses varying degrees of shock were encountered in the surviving animals. In the Benadryl-treated group significant protection was afforded, in that 2.0 mg/kg of histamine, 5 times the amount necessary to kill all unprotected animals, were required to produce 100% mortality. Some degree of shock was encountered in practically all animals which survived lesser doses. The animals receiving Pyribenzamine showed a considerably higher degree of protection against the lethal effects of histamine. Little evidence of shock and no deaths were observed up to 2.0 mg/kg, while 15.0 mg/kg of histamine were required to kill all animals. The data obtained would indicate that Pyribenzamine is approximately 6 to 7 times more active than Benadryl in preventing fatal histamine shock in guinea pigs.

Anaphylactic Shock. Seventy-two male guinea pigs weighing from 300 to 400 g were passively sensitized by the subcutaneous injection of 0.5 cc of rabbit anti-horse serum

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[8] Friedlaender, A. S., *Am. J. Med. Sc.*, 1946, in press.

[9] Friedlaender, S., and Feinberg, S. M., *J. Allergy*, 1946, **17**, 129.

[10] Arbesman, C. E., Koepf, G. F., and Miller, G., *J. Allergy*, 1946, in press.

* Benadryl was supplied by Parke-Davis and Co., Detroit, Mich.; the Pyribenzamine was furnished by Ciba Pharmaceutical Products, Inc., Summit, N.J.

TABLE I.
Protective Effect of Benadryl and Pyribenzamine Against Histamine Shock in Guinea Pigs.

Histamine I.V. mg (base)/kg	Control group Mortality		Group receiving Benadryl 3 mg/kg Mortality		Group receiving Pyribenzamine 3 mg/kg Mortality	
	Total deaths / Total used	%	Total deaths / Total used	%	Total deaths / Total used	%
.03- 0.1	0/3	0				
0.2	2/6	33				
0.3	3/6	50	0/5	0		
0.4	10/10	100	2/7	29	0/6	0
0 8 - 1.6			2/6	33	0/5	0
2 0			6/6	100	0/4	0
2.4 - 3.2					1/6	16
3.6 - 6.8					2/10	20
7 -10					3/9	33
11 -13					5/8	62
15					10/10	100

TABLE II.
Protective Effect of Benadryl and Pyribenzamine Against Anaphylactic Shock in Guinea Pigs.

Amount of drug used	mg/kg	No. of animals used	Survived	Died
None		12	1	11
Benadryl	1	10	5	5
	2	10	7	3
	3	10	10	0
Pyribenzamine	1	10	4	6
	2	10	7	3
	3	10	10	0

(Table II). After 48 hours, the intravenous injection of 1 cc of horse serum in the penile veins of 12 animals in this group resulted in typical fatal anaphylactic shock in 11. One animal manifested severe symptoms with recovery. The remaining animals were divided into 6 groups of 10 each, and given intraperitoneal injections of 1, 2, or 3 mg/kg of Benadryl or Pyribenzamine 15 minutes before the intravenous administration of 1 cc of normal horse serum. A significant degree of protection was afforded by 1 mg/kg of either drug. Two mg/kg gave somewhat increased protection, while 3 mg/kg of Benadryl or Pyribenzamine protected against fatal anaphylactic shock in all animals tested. Some manifestations of anaphylaxis were observed in the majority of the surviving guinea pigs. Subject to the limitations of the above experiment, this might indicate that there is no essential difference in the anti-anaphylactic activity of the 2 drugs under study.

Discussion. The protective effect of Benadryl and Pyribenzamine against histamine and anaphylactic shock is striking and in accord with the theory that histamine plays a role in anaphylaxis. Pyribenzamine on a weight basis has a greater protective effect against histamine than does Benadryl as manifested by the large increase in the LD_{100} of histamine in Pyribenzamine-treated animals. On a weight basis the 2 drugs appear to have an equal effectiveness against anaphylactic shock. This apparent discrepancy may be due to the fact that the maximum amount of histamine liberated during anaphylaxis in the guinea pig is of the order of 0.4 mg/kg, at which dose the 2 drugs are nearly equally effective. There is of course the consideration that phenomena other than the liberation of histamine may account for some difference between the protective effect of chemical agents against histamine shock on the one hand, and against anaphylactic shock on the other.

Summary. The LD_{100} of histamine was

first determined in a control group of guinea pigs. It was found that approximately 5 times this amount was required to kill all animals previously treated with 3 mg/kg of Benadryl, while 35 times the lethal dose of histamine was necessary to produce 100% mortality in animals receiving 3 mg/kg of Pyribenzamine. No apparent difference was discernible between the 2 drugs in preventing anaphylaxis in passively sensitized guinea pigs. One mg/kg of either compound gave significant protection against a shocking dose of antigen, while 3.0 mg/kg prevented fatal anaphylaxis in all animals tested.

15375

Bed-side Agglutination Test with Whole Blood for Rapid Diagnosis of Tularemia.*

RAÚL M. TOVAR. (Introduced by M. R. Castaneda).

From the Department of Medical Research, General Hospital, Mexico D. F.

The agglutination test has been the most practical method for the laboratory diagnosis of tularemia. McCoy and Chapin[1] showed the presence of agglutinins in the serum of patients infected with *B. tularense* and Francis[2] applied the agglutination test to the serological diagnosis of the infection. Recently Damond and Johnson[3] described what they call the "shake method" of agglutination by which it is possible to accelerate the reaction and read the results in 3 minutes.

Considering the possibility of a further improvement by using the method recommended by Castaneda and collaborators for typhus fever[4] and brucellosis,[5] we prepared a concentrated antigen conveniently stained and used either with whole blood as a bed-side test or with serum as in the case of the so-called rapid antigens developed by Huddleson[6] and Welch.[7]

Preparation of the Antigen. The strain of *B. tularense* No. 408, obtained by courtesy of Dr. R. R. Parker from the Rocky Mountain Laboratory of Hamilton, Montana, was used for the preparation of the antigen. The culture medium, recently described,[8] consisted briefly in a concentrated liver infusion with cystine, glucose, sodium chloride, peptone and agar, without blood or hemoglobin and distributed in Roux's bottles. Each bottle was inoculated with a concentrated emulsion of *B. tularense* and after 72 hours of incubation at 37°C the organisms were emulsified with isotonic saline containing 10% formaline (40%), filtered through wet cotton and left at ordinary temperature for 72 hours. The emulsion was centrifuged and the supernatant fluid was discarded; the organisms were emulsified in a small amount of isotonic saline. The concentration of the emulsion was standardized in order that one-tenth of antigen diluted with 10 cc of saline gave a turbidity corresponding to No. 3 of McFarland's Nephelometer. When the concentration of the antigen was adequate, enough aqueous solution of methylene blue was added to stain the antigen to a deep blue color. After 24 hours the antigen was centrifuged at high speed and the supernatant fluid was discarded.

* This work was aided by grants from the University of Mexico and Eli Lilly Co. of Indianapolis, Indiana.

1 McCoy, G. W., and Chapin, C. W., *J. Infect. Dis.*, 1912, **10**, 61.

2 Francis, E., *Medicine*, 1928, **7**, 411.

3 Damond, S. R., and Johnson, M. B., *J. Lab. and Clin. Med.*, 1944, **29**, 976.

4 Castaneda, M. R., Silva, R. G., and Monnier, A., *Rev. Med. del Hosp. General*, 1940, **8**, 382.

5 Castaneda, M. R., "Brucelosis," First edition, *Medicina*, 1942.

6 Huddleson, I. F., Tech. Bull. No. 123, Mich. Agric. Exp. Station, 1932.

7 Welch, H., and Stuart, C. A., *J. Lab. and Clin. Med.*, 1936, **21**, 411.

8 Tovar, R. M., *Rev. del Inst. de Salubridad y Enf. Trop.*, 1945, **6**, 181.

The stained organisms were emulsified in a small amount of isotonic sodium citrate solution (1.1%), containing merthiolate to a concentration of 1:5000.

Samples of the concentrated material are diluted to various proportions with isotonic citrate and each dilution tested for specificity and sensitivity using samples of serums with titers previously determined by the tube agglutination method. The test is performed mixing a drop of antigen with a drop of serum in a slide, and the sample giving definite agglutination within one minute with a serum of 1:50 titer is selected as a basis for further titrations. Then the antigen is tested for sensitivity which is made comparing the titer of a serum determined by the standard tube agglutination method and the results of tests performed on a glass slide using the rapid antigen following the method of Huddleson for brucellosis.[6]

The specificity of the antigen has been determined with a few available sera from patients suffering from tularemia, 2 from Mexico and 4 obtained by courtesy of Dr. Parker (Hamilton, Montana); all of them were confirmed cases. We also used sera from guinea pigs and rabbits experimentally infected with a non-virulent *B. tularense* strain. The inactivity of the antigen when mixed with normal sera or with serum from persons suffering from various infections, not including tularemia or brucellosis, was demonstrated performing 1600 comparative tests using the rapid antigen and the tube agglutination method.

When the antigen is found satisfactory it is submitted to tests with whole blood of guinea pigs or rabbits infected with *B. tularense*.

Technic of the test. Bed-side test with whole blood. The agglutination test with whole blood is performed at the bed side, mixing on a clean slide the antigen with blood taken by puncture of the ear or from the finger. The amount of antigen is that carried by a wire loop of 4 mm diameter and that of the blood is what is taken with a 2 mm loop. The slide is moved to and fro and the effect observed in front of a window or a bright light. The positive test appears within one minute, consisting of a definite separation of colors (blue of the antigen and red of the blood) and the immediate formation of clumps of antigen, which because of the rotation of the mixture, have a tendency to be accumulated in the periphery forming a blue ring. In the positive reactions there are different intensities that we called 1_t, 2_t, 3_t and 4_t, according to the time of clumping and size of clumps of antigen; they are related to a low or high agglutinating titer of the blood serum. The rapid reaction with whole blood is positive only in cases in which the agglutinating titer of the serum is above 1:100. In the negative tests there is neither color separation nor clumping of the antigen, the mixture remains homogeneous until drying.

The spontaneous clumping of red blood cells may be confused with a positive result for which it is necessary to perform a new test using the blood serum.

Rapid test with blood serum. The antigen has been satisfactory, used as a screen test, mixing a droplet of antigen with an equal amount of serum. When the test is positive there is an immediate formation of blue clumps of antigen and if it is negative the mixture remains homogeneous until drying. The screen test is positive when the agglutinating titer of the serum is above 1:20. The screen test with serum is particularly useful to pick up suspicious cases when a considerable number of samples is submitted to the laboratory. The positive serums are further tested by the rapid method following the technic of Huddleson and finally by tube agglutination using suitable emulsions of *B. tularense* to determine the agglutinating titer.

Results. The bed-side test with whole blood was performed with known cases of tularemia, suspicious cases detected by previous agglutination tests and blood of animals experimentally infected. The negative controls were normal persons or patients suffering from various diseases. We included a group of 100 cases of brucellosis, which according to Francis and Evans[9] produces a high percentage of cross-agglutination with *B. tularense*. Table I

[9] Francis, E., and Evans, A. C., *Pub. Health Rep.*, 1926, **41**, 1273.

TABLE I.
Bed-side Agglutination Test with Rapid *B. tularense* Antigen and Whole Blood.

No. of cases	History	Positive tests	%
100	Normal adults	0	0
100	Adults suffering from various infectious diseases	0	0
100	Brucellosis patients	10	10
30	Serological reactors to *B. tularense**	30	100
2	Confirmed cases of tularemia	2	100
20	Guinea pigs and rabbits experimentally infected	20	100

* Reactors determined by tube agglutination tests.

summarizes the results of the tests.

It may be seen that in spite of the few positive cases, the results of the test were very significant. The test was repeated many times in each case. The positive tests were clear cut in the 30 persons in whom it was found that the serum had a significant titer of agglutinins for *B. tularense*. In regard to the 10 cases of cross-agglutination found in the group of 100 patients suffering from brucellosis this is not surprising because of the previous findings referred to above. These cases of cross-agglutination can be readily differentiated by means of selective agglutination reported elsewhere.[10]

In the guinea pigs experimentally infected with tularemia the test was positive after the 7th day of inoculation and remaining positive during 7 months until discarded.

All individuals showing a positive bed-side agglutination test with whole blood, including the 10 cases infected with *Br. melitensis*, gave allergic skin reaction when injected by intradermal route with "Tulargen,"[10] an extract obtained by grinding *B. tularense*. The opsonocytophagic test performed with killed *B. tularense* as antigen and blood of the same persons, was positive in all cases.

Summary. A bed-side test for the rapid serological diagnosis of tularemia is described. The test is performed mixing a droplet of a concentrated suspension of *B. tularense* with a droplet of whole blood taken from the ear or from the finger of the patient. The minimum agglutinative titer of the patient's serum must be 1:100 to be detected by the rapid test. The antigen may be used for screen tests with blood serum.

[10] Tovar, R. M., *Medicina*, 1945, **25**, 331.

15376

Some Pharmacological Properties of the Monoanilide of Aconitic Acid.

R. M. FEATHERSTONE AND EMIL K. VENTRE. (Introduced by E. G. Gross).

From the Department of Pharmacology, College of Medicine, The State University of Iowa, Iowa City, and the Agricultural Chemical Research Division, U. S. Department of Agriculture, Baton Rouge, La.

The monoanilide of aconitic acid was prepared by one of us* from the *cis* anhydride according to the method of Nau, *et al.*[1] This compound was one of a series of derivatives of aconitic acid which were being tested as nonaqueous solvents for medicinal agents. The anilide group is attached to one of the carboxyl groups connected to the unsaturated carbon linkage. It was deemed of interest to compare the toxicity and the analgesic and antipyretic actions of this compound with those of acetanilide.

* Emil K. Ventre.

[1] Nau, C. A., Brown, E. B., and Bailey, J. R., *J. A. C. S.*, 1925, **47**, 2596.

Toxicity. Forty rats weighing approxi-

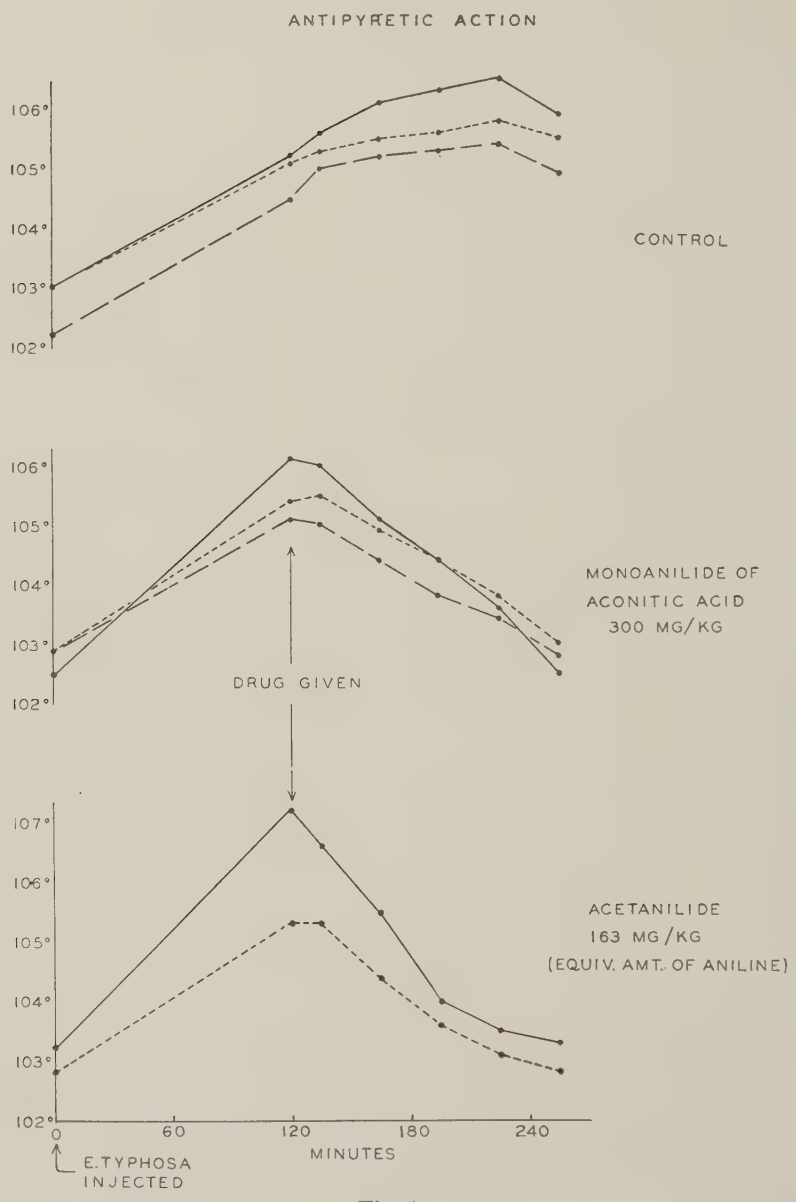

Fig. 1.

mately 200 g each were used in determining the LD_{50} of the compound when administered in suspension through a stomach tube. Twenty of the rats were used in a preliminary series of experiments to learn the approximate LD_{50} and the other 20 were divided into groups of 4 for each of 5 dose levels between 3.8 g/kg and 4.2 g/kg. The data in Table I support the assumptions that the toxicities of the monoanilide of aconitic acid and acetanilide are due primarily to the aniline in the molecules and that the 2 compounds have similar toxicities on a molecular basis.

Analgesic Action in Dogs. Three dogs were given on different days oral doses of 50 mg/kg of the monoanilide of aconitic acid and 27.25 mg/kg of acetanilide—amounts having equal weights of aniline. The rise in pain threshold was measured in each case

TABLE I.
Toxicity.

Compound	LD_{50}	Aniline %	Aniline g
Monoanilide of aconitic acid	4.0	37	1.5
Acetanilide	2.42	68	1.6

by the method of Hardy, *et al.*,[3] as adapted for use with animals. The data in Table II show that these 2 compounds in the doses given were essentially of equal efficiency in raising the pain threshold of dogs.

TABLE II.
Analgesic Action

	Rise in pain threshold			
	Monoanilide		Acetanilide	
Dog No.	Peak rise, %	Time, min	Peak rise, %	Time, min
1	17	50	20	65
2	18	55	18	60
3	17	60	16	60

[2] Sollman, T. H., and Hanzlik, P. J., *Fundamentals of Experimental Pharmacology*, J. W. Stacey Co., San Francisco, 1939, 241.

[3] Hardy, J. D., Wolff, H. G., and Goodell, H., *J. Clin. Invest.*, 1940, **19**, 649.

Antipyretic Action in Rabbits. An elevation in body temperature was produced in 8 rabbits by injecting intravenously 1 cc of a preparation of killed *Eberthella typhosa* containing approximately 10^7 organisms/cc. Rectal temperatures were measured before the injection and at intervals afterward. Either the monoanilide of aconitic acid or acetanilide was administered orally to groups of rabbits after an elevation of 2-4 degrees in body temperature was noted, usually 2 hours after the initial injection. The resultant decreases in temperature are compared in Fig. 1 with the temperatures of a group of rabbits given no drug.

Summary. The monoanilide of aconitic acid appears to have no advantages over acetanilide as an analgesic or antipyretic agent. The activity of the monoanilide seems to be related to the aniline in the molecule, as is the case with acetanilide.[4] No additional pharmacological actions were noted which could be ascribed to the propene group of the aconitic acid.

[4] Michel, H. O., Bernheim, F., and Bernheim, M. L. C., *J. Pharm. and Exp. Therap.*, 1937, **61**, 321.

15377

Starch Reaction as Aid in Identification of Causative Agent of "European Blastomycosis."

J. MAGER AND M. ASCHNER. (Introduced by B. Zondek).

From the Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem.

The clinical picture and symptomatology of "European Blastomycosis" ("Torulosis") is variable, and unreliable as a basis of diagnosis. The differentiation of the etiological agent is beset with difficulties, and its cultural and morphological resemblance to contaminating non-pathogenic yeasts has given rise to much confusion in the literature of yeast infections. The diagnostic scheme outlined below may be helpful in this connection, especially to pathologists without training in mycology, as it avoids the use of refined morphological criteria.

While studying the biochemical properties of nonfermenting capsulated yeasts, we observed that a strain isolated from a case of torula-meningitis produces extracellular starch in special growth conditions.[1] The amount of starch produced is considerable and can easily be detected macroscopically when a plate culture of the organism is flooded with Lugol's iodine solution.

We have now extended our investigation

[1] Aschner, M., Mager, J., and Leibowitz, J., *Nature*, 1945, **156**, 295.

to 16 other strains of this pathogenic yeast and in all of them starch formation was observed in the growth conditions indicated below. The individual strains varied as to the intensity of starch production. In the majority of cases a positive reaction for starch could be obtained after 24 - 48 hours of growth. In some strains the formation of starch was weaker and delayed for 5-8 days. A large collection of other yeast genera was tested and none of them exhibited the phenomenon of extracellular starch production. A substance which stains blue with iodine may be found in cultures of Schizosaccharomyces,[2] but only intracellularly and in very limited amount. The production of extracellular starch seems to be specific for a group of nonfermenting capsulated yeasts of which *Torulopsis neoformans* is a representative. This group includes also nonpathogenic species[3] which can, however, be differentiated by testing the growth optimum. Benham has shown, that whereas pathogenic *Torulopsis neoformans* (*Cryptococcus hominis,* according to Benham's nomenclature) thrives at 37°C, nonpathogenic species of this group show optimum growth at 30°C and poor or no growth at 37°C.[2]

In the light of these findings we arrive at the following method for the identification of *Torulopsis neoformans:*

Yeasts isolated from cases suspected of blastomycosis should be transferred to a synthetic medium composed as follows:

$(NH_4)_2SO_4$	0.1%
$MgSO_4$	0.05%
KH_2PO_4	0.1%
Glucose	1%
Thiamine	0.2 μg per ml
Agar-agar	2.5%

Ammonium sulfate serves in this medium both as a source of nitrogen and as a regulator of the acidity necessary for production of starch.[1] Vitamins other than thiamine are not essential for cultivation of starch-producing yeasts in synthetic media.

After some days of incubation at 37°C, the plate is flooded with Lugol's iodine solution. In positive cases the whole streak of growth usually turns a deep blue. If, however, the amount of starch elaborated by the yeast is small, the reaction is perceptible only in isolated spots and after excess of iodine has disappeared. The extracellular character of the starch may be ascertained by examining with iodine the color of the agar beneath a growth-streak after the cells are scraped off from the agar surface. An alternative method is to cultivate the yeast in a liquid medium (composed as above), where optimal growth can be obtained by aeration or continuous shaking. After the cells have been separated by centrifugation, the supernatant is tested for starch.

According to our results, ability of extracellular starch formation combined with unimpaired growth between 37° and 40°C are features characteristic enough to classify a nonfermenting yeast as *Torulopsis neoformans* (Sanfelice) Lodder.[4,5]

Summary. A method for the identification of *Torulopsis neoformans* (syn. *Torula histolytica, Cryptococcus hominis*) is described.

The method is based on the ability of this organism to produce extracellular starch in special growth conditions.

We should like to express our thanks to Prof. Kluyver, Dr. Lodder, and Dr. Benham for their kindness in sending us a large number of different yeast cultures.

[2] Beijerinck, M. W., *Centralblatt f. Bakt. u. Parasitenk.*, 1897, **3**, 449.

[3] Benham, R. W., *J. Infect. Dis.*, 1935, **57**, 255.

[4] Lodder, J., 1934, *Die Anascosporogenen Hefen,* p. 152, Amsterdam.

[5] Lodder, J., *Mycopathologia,* 1938-9, **1**, 62.

15378

Histochemical Distribution of Alkaline Phosphatase in Dog Liver After Experimental Biliary Obstruction.

M. Wachstein and F. G. Zak. (Introduced by E. P. Pick).
From the Laboratories of Mount Sinai Hospital, New York City.

Gomori[1] was the first to notice that the bile capillaries in the liver of some species showed marked phosphatase activity. The constant increase of serum alkaline phosphatase after ligation of the bile ducts in the dog[2] and the fairly regular phosphatase activity of the bile capillaries in the dog liver suggested that a study be made of the behavior of alkaline phosphatase activity in the liver after ligation of the bile ducts.

Material and Methods. In 9 mongrel dogs the common bile duct was ligated under nembutal anesthesia after an initial biopsy had been taken. In 2 acute experiments the cystic duct also was ligated. Serum phosphatase activity was estimated with Bodansky's method[3] using a Leitz photocolorimeter. Gomori's method[4] as modified by Kabat and Furth[5] was used for the histochemical demonstration of alkaline phosphatase activity. Alternate sections were routinely incubated for 2 and 14 hours. When indicated, the incubation period was shortened to 30 and 60 minutes. No counterstain was employed.

Results. The changes in the dog liver after duct ligation as seen in slides stained with hematoxylin-eosin, were essentially similar to those previously described.[6,7] They consisted of hyperemia, atrophy of liver cells in the central fields, occurrence of bile thrombi, predominantly in the central fields and very occasional occurrence of small foci of necrosis. Bile pigment was seen in the liver cells mainly around central fields. Kupffer cells frequently contained bile pigment.

Normal Liver. (2-hour incubation). With the exception of bile capillaries and larger bile ducts, there was only very faint phosphatase activity in all other structures. The cytoplasm of the liver cells took on a faint grayish color. Most of the nuclei could be recognized due to some staining of the nuclear membrane and of the nucleoli. Only occasionally was there activity in the endothelium of the sinusoids near the periportal areas. Larger bile ducts in the periportal areas showed marked phosphatase activity. The bile capillaries were outlined as fine, black, lines sometimes showing a definite lumen (Fig. 1). They showed frequently, the well-known tortuous appearance. In some areas, they were seen between 2 liver cell cords (trabecular), in others they formed networks apparently surrounding single liver cells. From the trabecular capillaries intercellular branches went off at various angles. On cross section, they appeared as small round black dots or circles. Very occasionally, the transition of a capillary into a bile duct could be seen. The activity of the bile capillaries was somewhat more distinct near the periportal fields than around central areas. There was, however, some variation in different livers.

Normal Liver. (14-hour incubation). The staining of the cytoplasm and of the nuclei became more intense. The activity of the sinusoidal lining as well as of their nuclei became marked. Connective tissue cells and occasional mononuclear cells in the periportal fields showed also some activity. The intensification of the reaction was moderate in

1 Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

2 Armstrong, A. R., King, E. F., and Harris, R. I., *Canad. M. A. J.*, 1934, **31**, 14.

3 Bodansky, A., *Am. J. Clin. Path.*, Techn. Suppl., 1937, **1**, 51.

4 Gomori, G., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 23.

5 Kabat, E. A., and Furth, J., *Am. J. Path.*, 1941, **17**, 303.

6 Ogata, T., *Beitr. Z. Path. Anat. u. Z. Allgem. Pathol.*, 1913, **55**, 236.

7 Hiyeda, D., *Beitr. Z. Path. Anat. u. Z. Allgem. Pathol.*, 1925, **73**, 541.

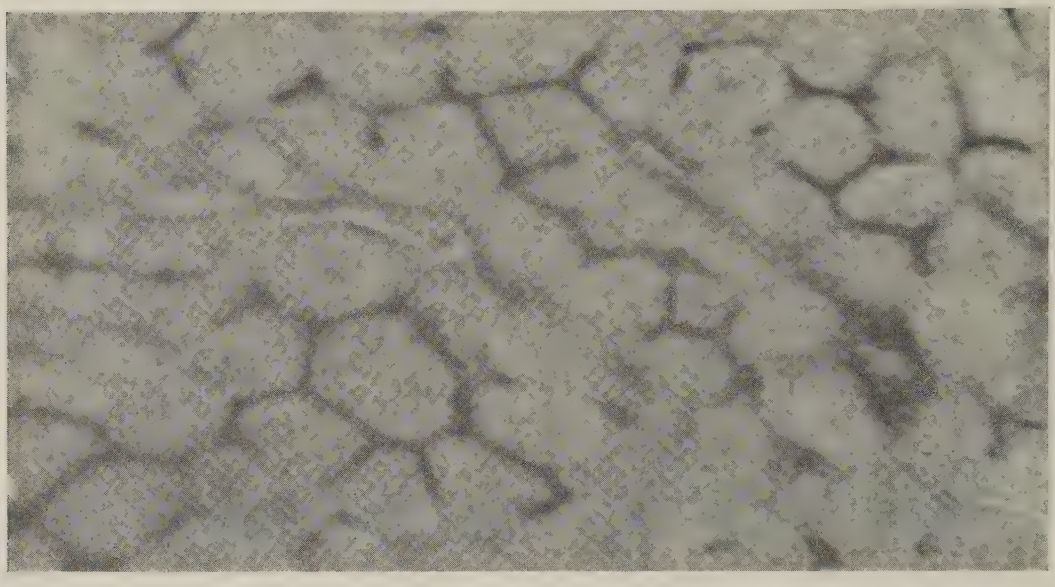

FIG. 1.
Alkaline phosphatase activity in the normal dog liver. The bile capillaries show prominent staining. Incubation in the substrate mixture for 2 hrs. × 800.

some, quite marked in other livers. In the latter group, the bile capillaries also showed stronger staining and occasionally their outline became hazy. The concomitant staining of the other structures made the activity of the bile capillaries less conspicuous.

Effect of Duct Ligation. (3-4 days). Two dogs died after 3-4 days because of extensive wound infection. One was killed 4 days after ligation. This dog showed a rise of blood serum phosphatase from 2.4 to 43.2 units. In sections incubated for 2 hours a considerable number of bile capillaries were wider than normal. Some showed a hazy outline. There was some increase in cytoplasmic phosphatase. After longer incubation, this increase became more striking and the cytoplasm showed many fine, black granules. The 2 dogs that died showed very marked dilatation of the bile capillaries which were considerably widened and not clearly demarcated, from the surrounding cytoplasm. In addition, considerable increase in cytoplasmic activity was seen.

One Week. One dog was killed after 8 days. Serum phosphatase rose from 1.1 to 47.6 units. In sections incubated for 2 hours, there was very marked dilatation of the bile capillaries which stood out much clearer than in the biopsy. Many of these showed a distinct lumen. The demarcation from the cytoplasm was not sharp in a large number of these capillaries. The cytoplasm showed increase in activity somewhat more pronounced around the periportal fields. The bile ducts in the periportal fields showed marked increase in activity and their lumen was filled by intensely dark staining material. After longer incubation, the reaction became so intense that the structures could hardly be differentiated.

Two Weeks. Two dogs were killed after 2 weeks. In one the serum phosphatase rose from 1 to 36 units. In this dog, dilatation of the bile capillaries occurred predominantly at the periportal fields. The second dog showed very widespread dilatation of the bile capillaries. While some of them still showed their clear outline, many became very irregularly demarcated and hazy. The increase in cytoplasmic activity was very conspicuous.

Three Weeks. One dog was killed 3 weeks after ligation. His initial serum phosphatase of 1.4 units rose to 27.6 units in 8 days, and to 32.2 units in 14 days. The increase in phosphatase activity was so intense that sections incubated longer than one hour could

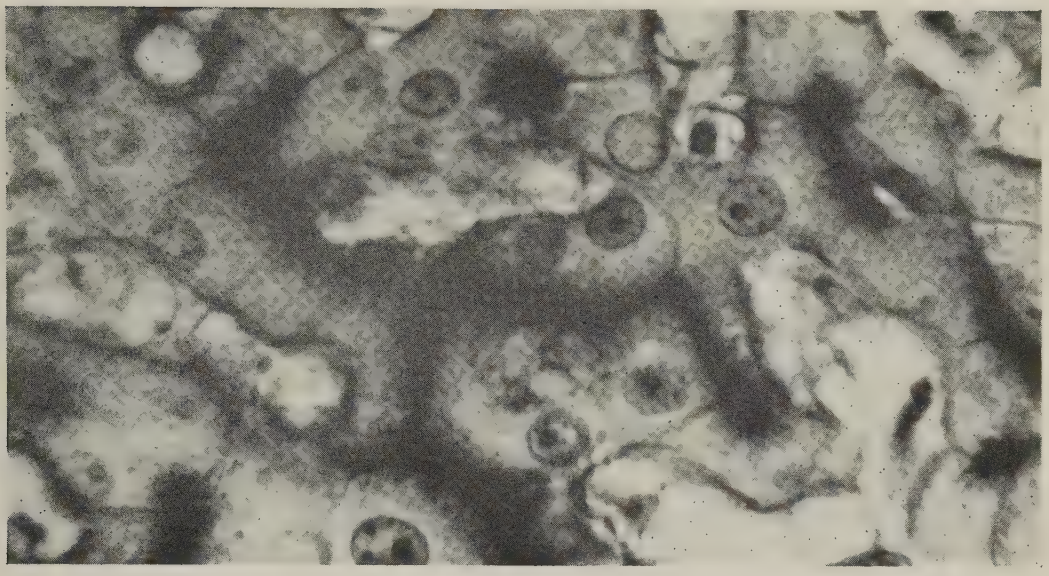

Fig. 2.
Alkaline phosphatase activity in the liver of a dog, 3 weeks after ligation of the common bile duct. Conspicuous widening of many bile capillaries. Incubation in the substrate mixture for 30 minutes. ×800.

not be used. Widening of the bile capillaries was extremely marked in many areas (Fig. 2). It appeared as if large amounts of phosphatase were accumulated in liver cells near the bile capillaries. In this way, the width of the bile capillaries, on cross section, appeared to be 4 to 5 times larger than normal. Occasionally, a lumen could be made out. In contrast to the conspicuous changes in many of the capillaries, some were of fairly normal appearance.

Acute Experiments. In 2 dogs after initial biopsy, the cystic and common ducts were ligated and biopsies were taken after 30, 60, 90, and 120 minutes. No changes were seen in serum phosphatase in one of the dogs in which this examination was carried out. After one hour the bile capillaries stood out somewhat more distinctly and showed occasional light dilatation. There was also a very slight increase in cytoplasmic activity.

Comment. The source of the increased amount of alkaline serum phosphatase which occurs in obstructive and to a lesser degree in parenchymatous jaundice is still controversial.

The accumulation of phosphatase around bile capillaries is in marked contrast to the diffuse increase of enzymatic activity which occurs in the liver of protein depleted mice and rats and to a lesser degree in starvation.[8] It can best be interpreted as retention of alkaline phosphatase in liver cells caused by external obstruction. No significant increase in alkaline phosphatase activity is seen, on the other hand, in necrotic liver cells.[8] The behavior of the histochemically demonstrable phosphatase activity under various experimental conditions therefore, supports the opinion of those who believe[9,10] that the increase of serum phosphatase in liver damage is due to disturbed excretion but not to increased production of alkaline phosphatase in the liver cells.[11]

Summary. Bile capillaries show distinct phosphatase activity in the normal dog liver. This property can be used for their microscopic demonstration. After ligation of the common bile duct there is not only marked

8 Wachstein, M., *Arch. Path.*, 1945, **40**, 57.

9 Armstrong, A. R., and Banting, F. G., *Canad. M. A. J.*, 1935, **33**, 243.

10 Maddock, S., Schmidt, G., and Thannhouser, S. F., *Federation Proc.*, 1942, **1**, 181.

11 Bodansky, A., *Enzymologia*, 1937, **3**, 258; Proc. Soc. Exp. Biol. and Med., 1939, **42**, 800.

dilatation of the bile capillaries but also apposition of phosphatase around them which increases with the duration of the experiment. The changes in the distribution of alkaline phosphatase in histochemical preparations favor the assumption that the increase of serum phosphatase in liver damage is due to the inability of the liver cells to excrete the enzyme rather than to increased production in the liver itself.

15379

Morphologic Effects of DDT on Nerve Endings, Neurosomes, and Fiber Types in Voluntary Muscles.*

Eben J. Carey, Estelle M. Downer, Frances B. Toomey, and Eugene Haushalter.

From the Department of Anatomy, Marquette University School of Medicine, Milwaukee, Wisc.

The effect of DDT (2, 2 bis(p-chlorophenyl)-1,1,1-trichloroethane) on the voluntary neuromuscular apparatus in the living, intact animal is unknown. Lewis and Richards[1] state that DDT does not affect the growth of various cells in 7- to 8-day chick embryos and in a 1-day rat in hanging drop cultures. They are unable to explain the apparent paradox of the toxicity of DDT to intact, living animals and its non-toxicity on their isolated cells in hanging drop cultures. Yeager and Munson[2] have presented physiological evidence for a possible site of action of DDT in bringing about repetitive discharges of nerve impulses into the muscles of the roach, *Periplaneta americana,* which indicates that the site (or sites) referred to is that region of a nerve which lies between the origin of its fibers in the ventral nerve cord and the termination of its fibers in the leg, exclusive of the origin and the endings, that is, the myoneural junctions. Their results suggest that DDT can act more readily on motor than on sensory nerves and, further, that DDT stimulates motor nerves somewhere along their course between the cells of origin in the ventral nerve cord and the nerve endings in muscle.

The peripheral effects of DDT on the living, nerve-intact muscle, nevertheless, are visibly evident in chameleons and rats, in the persistent, involuntary, clonic contractions and occasional epileptoid convulsions and violent tremors of the entire voluntary musculature until complete flaccid or spastic paralysis and death occur. The purpose of this paper, therefore, is to demonstrate the striking morphologic changes observed in the voluntary neuromuscular apparatus of chameleons and rats during the transmission of a quantitative increase of neurogenic substances into the myoplasm of some muscle fibers affected by DDT.

Methods. A quick-killing emulsion[1] was prepared which contained 1% DDT, 8% olive oil, 1% gum arabic, and 90% Locke's solution; 2 cc of this emulsion was injected intraperitoneally into 48 white rats *(Mus norvegicus),* average weight 250 g, and 0.1 cc into 100 summer pseudo-chameleons *(Anolis caroliniensis),* average weight 3 to 8 g. Eight rats were selected at 12-hour intervals after the injection of DDT for microscopic study of the gastrocnemius muscle, sciatic nerve, and spinal cord. Various histologic technics were employed for the study of Nissl substance, myelin sheath, axis cylinders, motor end plates, and liposomes in muscle. Ten chameleons were selected also, but at 2-hour

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc., and the Baruch Committee on Physical Medicine.

[1] Lewis, W. H., and Richards, A. G., Jr., *Science,* 1945, **102**, 330.

[2] Yeager, J. F., and Munson, S. C., *Science,* 1945, **102**, 305.

intervals after the injection of DDT because of the more rapid action of DDT in the chameleon than in the rat. The same microtechnics were employed to study the muscles, peripheral nerves, and spinal cords of the chameleons as in the rat.

The lower extremities in 10 chameleons were quickly skinned and then immersed vertically in Locke's solution, 50°C, for 10 seconds. The muscles of the lower extremities, immediately upon immersion, manifested heat rigor. After immersion, the muscles were quickly excised and prepared for microscopic study. The bloodless muscles of the lower extremities from 10 DDT rats were excised after the muscles were perfused, through the arch of the aorta, with physiological salt solution until clear fluid poured out of an incision in the right auricle of the heart. The muscles were dehydrated *in vacuo* and fed to 10 rats. Seven of the 10 rats manifested DDT toxicity within 3 days. Two hundred fifty mg of dehydrated and triturated muscle, previously perfused, from rats killed with DDT, were suspended in 10 cc of olive oil. Five-tenths of one cc of this suspension were injected intraperitoneally into each of 20 chameleons. Fourteen manifested DDT toxicity within 48 hours and 6 were unaffected. The muscles, peripheral nerves, and spinal cord from 10 normal white rats and 10 chameleons quickly killed by ether were excised, subjected to the microscopic methods of preparation enumerated above, and used as controls. The microscopic observations reported in this paper will be confined dominantly to those demonstrated by the gold technic previously described.[3]

Tests were made *in vitro* of the chemical reactions of 1% gold chloride with 1% acetylcholine chloride, and of 1% gold chloride with 1% choline chloride. Precipitates of acetylcholine aurichloride and choline aurichloride, respectively, were formed.[4] When this reaction occurred in capillary glass tubes with an inside diameter of 100 μ, evanescent cross striations of periodic precipitation formed, which subsequently disappeared in a uniform diffusion of granules. One percent osmic acid formed a precipitate both with 1% acetylcholine chloride and 1% choline chloride. One percent gold chloride also produced a precipitate with 1% alcoholic solution in each of the following: oleic, palmitic, and stearic acids. There was likewise an interaction between gold chloride and lecithin, and a very strong interaction in the reduction of gold by 1% ascorbic acid and by 1% thiamine hydrochloride.

Results. After the intraperitoneal injection of DDT in the rat, death occurred within 12 to 72 hours, and in the chameleon within 6 to 24 hours. Forty-one of the 50 rats had definite chromodacryorrhea due to overstimulation of the harderian glands which secreted the red-brown porphyrin pigment. This was comparable to the toxic effects of acetylcholine. All the rats had intense salivation, involuntary passage of feces and urine, and clonic, involuntary contractions and tremors of the voluntary muscle, which were the usual physical manifestations of toxicity due to DDT in the rat. In the chameleon the pigmentation of the skin usually changed from green to dark brown, and there were persistent, intermittent contractions of the voluntary muscles. When the chameleons in the terminal stages of DDT toxicity were skinned, there was a continuation of intermittent and incoordinate muscle action that resembled both fibrillation and fasciculation of denervated muscle. This was likewise observed in isolated muscles for 10-20 minutes after excision at 22°C.

The salient microscopic morphologic changes in 0.5% to 15% of the motor innervations in 100 rat and 200 chameleon gastrocnemius muscles after toxicity with DDT (Fig. 2, 3, and 6), in contrast to the normal control muscles (Fig. 1, 4, and 5) in the teased whole muscle fibers and cross sections, were the following: (1) progressive depletion of some epilemmal axons of auriphilic substance and demyelinization of some peripheral nerves; (2) centrifugal discharge from the motor end plate of these auriphilic neurosomes into the myoplasm either as elongated streamers, serial beads, or large elongated masses; (3) the appearance of this auriphilic

[3] Carey, E. J., *Anat. Rec.*, 1941, **81**, 393; *Am. J. Path.*, 1942, **18**, 237.

[4] Loach, J. V., *J. Physiol.*, 1934, **82**, 118.

FIG. 1 to 3.

Photomicrographs × 200. Normal innervation, Fig. 1, discharge of massive neurosomes from end plates by DDT, Fig. 2 and 3. There is progressive depletion of auriphilic substances in the epilemmal axons, Fig. 2 and 3, contemporaneous with the appearance of the auriphilic neurosomes in the myoplasm. Legend: epa, epilemmal axons; hya, hypolemmal axons; NS, neurosomes. Gold chloride; teased whole gastrocnemius muscle fibers; chameleon.

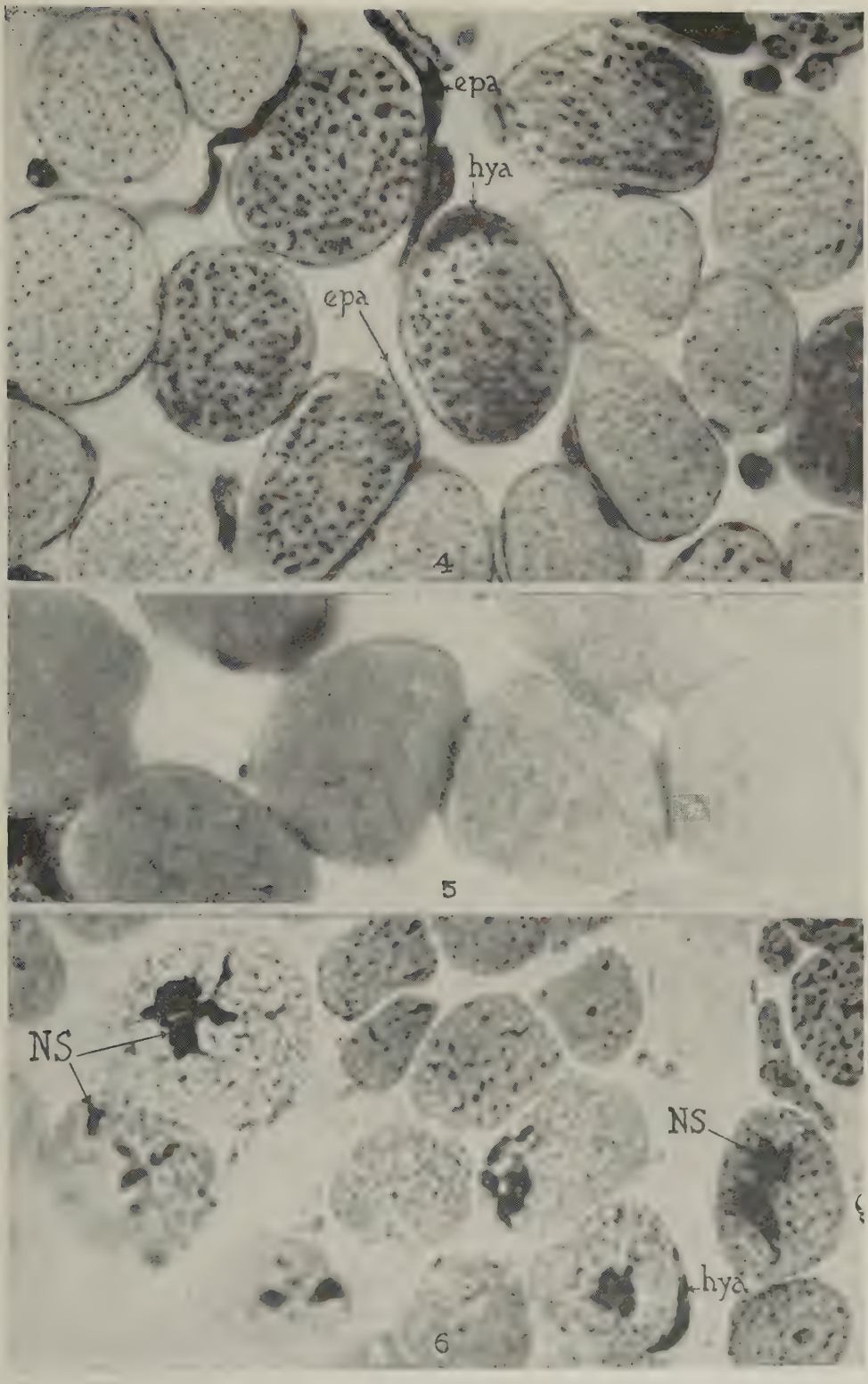

FIG. 4 to 6.

Photomicrographs × 500. Normal coarsely and finely granular muscle fibers, Fig. 4; and agranular fibers, Fig. 5; and agglutination of neurosomes, NS, after DDT, Fig. 6. Legend: epa, epilemmal axons; hya, hypolemmal axons. Gold chloride; cross sections, gastrocnemius muscle, chameleon.

neurogenic substance in the myoplasm of the muscle fiber by jet-pump action from, and in anatomical continuity with, the motor end plate (Fig. 2 and 3); (4) the subsarcolemmal position of the agglutinated islands of augmented auriphilic substance, seen in whole teased muscle fibers (Fig. 3) and in the cross sections (Fig. 6); (5) an acute atrophy occurring in some muscle fibers affected by DDT (Fig. 6); (6) disappearance of the motor end plates in 10% to 30% of the biopsied gastrocnemius muscles, from 10 chameleons and 10 rats, during the terminal stage of DDT paralysis.

There was an acute atrophy of the muscle fibers in some places. In some muscle fibers dispersion of the granules was lost completely. The granules were agglutinated in large masses in localized spots in some muscle fibers; and this was comparable to the loss of granules in some parts and aggregation in other parts of the specific granular muscle fiber observed 10-18 days after denervation,[5] and after 12 days of total starvation,[6] except water, in the rat. In these denervated gastrocnemius muscles the fine neurosomic granules of Kühne disappeared around the degenerated nerve endings about the 72nd hour after section of the sciatic nerve. There were, however, centrifugal discharges of large, coarse granules into the muscle from the greatly altered nerve terminals, until the distal stump of nerve was depleted of degenerated axonic and myelin substances. This final depletion stage of the distal stump of the sectioned nerve varied in individual rats from 10 to 18 days. The specific structure and dispersal of granules, therefore, in the dark muscle fibers appeared to depend upon the intact nerve endings and normal function of the innervation of muscle.

In the normal muscle fiber of the chameleon there was variation in the distribution of the granular and agranular muscle fibers. The agranular muscle fibers were increased in number by heat rigor of short duration produced by submerging the skinned chameleon in Locke's solution for 10 seconds at 50°C. The agranular fibers produced by this short duration of heat rigor were comparable to the normal agranular fibers (Fig. 5). In heat rigor 90% to 96% of the muscle fibers were agranular, whereas in the normal only 40% to 75% were agranular, 20% to 58% finely granular, and only 2% to 5% coarsely granular. This differential count was based on the observations of 2000 fibers in each of 20 gastrocnemius muscles from 10 chameleons: a total of 40,000 fibers. Although the muscle fibers with greatest diameter were usually pale and agranular, this was not an invariable finding. In the winter animal there was a number of pale fibers with very fine granules, or agranular, that possessed small fiber diameters. Some other medium-sized muscle fibers of the dark type were packed with coarse granules. The normal granules of the muscle fibers varied in diameter from 0.2 to 10 μ.

Some of the auriphilic granules formed elongated bipolar streamers in relation to the muscle nuclei. The granules in the muscle fiber, therefore, were dual in origin: (1) neurosomes discharged from their motor nerve endings and diffused in the myoplasm; and (2) granules discharged from the 2 terminal poles of each of the multiple elongated nuclei in the protoplasm of the muscle. In some places the granules related to the muscle nuclei and the neurosomes continuous with the nerve endings appeared to intermingle and coalesce to form one elongated granular mass. There were many nuclei located in the center of the granular fibers whereas most of the nuclei were located under the sarcolemma in the agranular muscle fibers.

The abnormal auriphilic masses of neurogenic substance produced by DDT were sometimes as long as 450 μ and had in some places (Fig. 3 and 6) a diameter of 50 μ. The normal granules and the abnormal masses of auriphilic neurogenic substance were inconstant in their refractive properties, size, shape, number, and capacity to react to gold, silver, methylene blue, osmic acid, sudan III and IV, sudan black, and alkaline scarlet red. The part of a muscle fiber depleted of its gold staining substance was definitely

[5] Carey, E. J., Massopust, L. C., Haushalter, E., Sweeney, J., Saribalis, C., and Raggio, J., *Am. J. Path.*, in press.

[6] Carey, E. J., *Anat. Rec.*, 1942, **82**, 403.

agranular when there was an aggregation of this substance produced by DDT in another part of the same fiber. The massive aggregation of auriphilic substance in the myoplasm of the voluntary muscle fiber was in anatomic continuity in many instances with the auriphilic substance of the hypolemmal and epilemmal axons undergoing depletion by the action of DDT. That at least some of the auriphilic masses were neurogenic in origin is proved by the following microscopic evidence: (1) there is frequently a continuous anatomic relationship between this auriphilic substance in the muscle and the nerve ending; (2) there is a similarity of reaction of this substance in nerve and muscle with gold; (3) there is an excessive and massive accumulation of this auriphilic substance in muscle parallel with the exhaustion of the nerve supply of its auriphilic substance.

Discussion. The mechanism of production of the granular and agranular muscle fibers is still unknown. This fact has been pointed out in the relatively recent excellent reviews and observations by Cobb,[7] Needham,[8] Hines,[9] Hinsey,[10] Denny-Brown,[11] and Tower.[12] The histologist uses various synonyms dependent upon observations made on fresh muscle or on those made after chemical alteration by fixatives and staining reactions for the "granular and agranular" muscle fibers, respectively, as follows: "dark and light or pale," "opaque and clear," "plasmic and aplasmic," "red and white," muscle fibers. Grützner[13] stated that all granular muscle fibers are red muscle fibers and that the agranular are white muscle fibers. Starling[14] claimed that all striated muscles of higher vertebrates are microscopically mixed muscles and contain both red and white types of muscle fibers. It was demonstrated by Knoll[15] that the granules in the small muscle fiber can be stained with osmic acid and gold. Kölliker,[16] Albrecht,[17] and Bell,[18] identified these granules as lipoid in nature, basing their identification on reactions with osmic acid and fat stains such as sudan III and scarlet red. However, we present evidence in this paper that choline and acetylcholine likewise react strongly with both osmic acid and gold chloride.

Krause[19] stated that the small granular muscle fiber was a young form that eventually grew into the adult large agranular fiber. Bonhöffer[20] studied the same muscle at different ages and found the distribution of the granular and agranular fibers was about the same at different periods in development. Schaffer[21] reported that the arrangement of the coarse myofibrils was without order in the granular fiber whereas there was more regularity in the distribution of the fine myofibrils in the agranular fiber into so-called areas of Cohnheim, in cross sections, or columns of Kölliker, in longitudinal sections. The so-called sarcoplasm was greater in red granular fibers than in white agranular muscle fibers according to Schaffer.

Experimental evidence is presented here which supports the assumption that there is a periodic shift in the location of the muscle nuclei in relation with the phase of the cycle of metabolism stopped by gold impregnation.

7 Cobb, S., *Physiol. Rev.*, 1925, **5**, 518.

8 Needham, D. M., *Physiol. Rev.*, 1926, **6**, 1.

9 Hines, M., *Quart. Rev. Biol.*, 1927, **2**, 149.

10 Hinsey, J. C., *J. Comp. Neurol.*, 1927, **44**, 87; *Physiol. Rev.*, 1934, **14**, 514.

11 Denny-Brown, D. E., *Roy. Soc. London, Proc.*, Series B, 1929, **104**, 371.

12 Tower, S. S., *Physiol. Rev.*, 1939, **19**, 1.

13 Grützner, P., *Pflüger's Arch.*, 1887, **41**, 256.

14 Starling E. H., *Principles of Human Physiology*, 5th ed., edited and revised by C. Lovatt Evans, Lea & Febiger, Phil., 1930, p. 139.

15 Knoll, P., *Z. f. Heilk.*, Berlin, 1880-81, **1**, S. 255; *Denkschr. d. k. Akad. d. Wissensch. Wien. Math. Naturw. Kl.*, Wien, 1891, **58**.

16 Kölliker, A., *Z. f. Wissensch. Zool.*, Leipzig, 1857, **8**, 311; *ibid.*, 1888, **47**, S. 689.

17 Albrecht, E., *Deutsche. path. Gesellsch.*, 1902, **5**, S. 7; *ibid.*, 1903, **6**, S. 63; *Ergeb. der allg. Path. u. Path. Anat.*, 1907, **11**, Abth. 2, S. 1166.

18 Bell, E. T., *Anat. Rec.*, 1910, **4**, 199; *Internat. Monatsch. f. Anat. u. Physiol.*, 1911, **28**, S. 297; *J. Path. and Bact.*, 1912, **17**, 147.

19 Krause, W., *Die Motorschen Endplatten der quergestreiften Muskelfasern*, Hannover, Hahn, 1869, pp. 192, and one plate.

20 Bonhöffer, K., *Pflüger's Arch.*, 1890, **47**, 125.

21 Schaffer, J., *Sitzungsb. d. k. Akad. d. Wissensch. Math.-Naturw. Kl.*, Wien, 1893, **102**, Abth. 3, S. 7.

It is suggested that the locations of the muscle nuclei are related to the mechanical effects of the chemical reactions produced by the discharge, hydrolysis, and disappearance of the granular neurosomes projected into the myoplasm from the nerve endings. It is likewise suggested that the fiber types in muscle appeared not to be fixed histologic structures but that they readily are transformed one into the other, dependent upon the phase of the metabolic cycle of nerve and muscle in which the tissue ceased activity by the impregnation with gold. The periodic changes in the histochemistry of muscle appeared to determine the granular and the agranular types of muscle fibers independently of the gross color of the muscle. The observations reported in the chameleon in this paper are made upon the grossly pale reptilian muscle, and the granular fibers have no relation to the so-called "red" muscle.

The same axis cylinder may divide and end in 2 motor end plates, one retracted in the granular muscle fiber and the other expanded in the agranular muscle fiber. This is suggestive evidence that the same axis-cylinder of a nerve, upon division, may terminate in muscle fibers fixed in different phases of either the discharge or hydrolysis of neurosomes in the metabolic cycle of nerve and muscle. The greater number of granular fibers found in the slower than in the faster reacting muscle is assumed to be related to the differential speed of metabolism and response to the chemical irritation produced by gold impregnation. If this assumption is true, then a greater number of granular fibers would be found in the slower than in the faster reacting muscles. These granular fibers, therefore, are not related to hemoglobin pigmentation and the adjective "red" should be discarded.

Denny-Brown[11] cut the ventral roots of the sciatic nerve in the cat. Two weeks later he found that the distribution and depth of staining of dark and light fibers in the gastrocnemius and soleus had remained the same on the operated side as on the control side. We have duplicated this experiment in 50 rats[5] and 10 cats. The definitive dark fibers disappear in from 10 to 18 days in the rat, and from 21 to 36 days in the cat. There is no way, based upon the use of either fat stains of alkaline sudan III, scarlet red, osmic acid or metallic impregnation with gold or silver, of clearly differentiating from one another acetylcholine, choline, lecithin, and other products of the breakdown of complex lipoids. Seventy-two hours after nerve section in the rat, acetylcholine is not identified by Dale, Feldberg, and Vogt,[22] yet there is a periodic discharge of some complex lipoids, in the form of very coarse granules, from the degenerated end plate, and these granules react to osmic acid, alkaline sudan III, and to gold chloride. This centrifugal discharge of the degenerated axonic and myelin material of the distal stump of the cut sciatic nerve into the denervated muscle continues until complete substantial exhaustion occurs. It is because of this fact, and species variation in degeneration of nerves, that Denny-Brown[11] did not observe any great change in the disappearance of the muscle granules in the narrow, dark fibers 14 days after nerve section in the cat. The dark, granular muscle fiber disappears when a sufficient length of time has elapsed after nerve section as well as after starvation. Denervation[5] results in a material, lipoidal exhaustion of the distal stump of the sciatic nerve, whereas starvation[6] produces either a partial or complete block in the substantial transmission of neurogenic substances in some fibers of the muscle. This is evident by the great enlargement of some of the nerve axons extrinsic to the sarcolemma[6] and the retraction of the hypolemmal axons and disappearance of the granules of Kühne.

The effect of DDT on the neuromuscular apparatus results in an augmented discharge and aggregation of neurosomes in the myoplasm. DDT produces dissociation of the neurogenic and myogenic substances in the voluntary muscle fiber. DDT likewise finally produces a chemical denervation of some muscle fibers microscopically detected by the disappearance of some of the motor end plates. DDT also causes demyelinization of some

[22] Dale, H. H., Feldberg, W., and Vogt, M., *J. Physiol.*, 1936, **86**, 353.

of the intramuscular medullated nerves.

The augmentation and aggregation of neurogenic substances in the voluntary muscle fibers affected by DDT parallels the abnormal response and subsequent paralysis through axonic exhaustion by chemical denervation of some muscle fibers. It is suggested that the normal dark and granular muscle fiber be designated as the neurosomic muscle fiber and that the light and agranular fiber be called the aneurosomic muscle fiber. This terminology is based on the hypothesis that there is a periodic discharge and disappearance of the neurogenic granules in the fibrillar protoplasm of the same voluntary muscle fiber observed at different periods of time, and the hypothesis itself is supported by the suggestive experimental evidence of the effects of DDT.

The observations reported in this paper are, in general, consistent with the views expressed, over one hundred years ago, by Doyère.[23] He observed the junction between the living nerve and muscle in the microscopic Tardigrade (Spallanzani) also called the "water bear." Its body is a transparent muscular bag. Doyère stated, page 346, that "the relation of the terminal nerve filaments with the muscles cannot be distinguished," and in Fig. 4, plate 17, he illustrates the junction of the granular nerve fiber and the granular muscle fiber as morphologically continuous.

Summary. The limited experimental evidence presented in this paper tends to support the statement that DDT increases the discharge of auriphilic neurogenic substances from some motor end plates into the myoplasm of some of the voluntary muscle fibers. These neurogenic substances form massive aggregates of neurosomes in parts of some voluntary muscle fibers. This results in a partial or complete dissociation of the neurogenic from the myogenic substances in the muscle fiber. Auriphilic granules are likewise found at the 2 poles of the elongated nuclei in the dark muscle fibers. The supporting evidence that some of the auriphilic granules and masses found after DDT toxicity are neurogenic in origin follows: (1) anatomical continuity of the auriphilic bodies in muscle with the auriphilic hypolemmal axons of the motor end plates; (2) similarity of staining reaction of the masses in muscle and axons of nerve endings with gold impregnation; (3) massive aggregation of auriphilic bodies in some muscle fibers contemporaneous with the centrifugal depletion of the related nerve axons and endings of their auriphilic substances. Presumptive evidence is presented that the normal fiber types in voluntary muscle are dependent, in part, upon the periodic alternation of the discharge and disappearance, by chemical action, of the neurogenic granules in the same muscle fiber at different time periods. The limited evidence also indicates that DDT poisoning may be produced in normal rats by feeding, and in chameleons by the intraperitoneal injection of the previously perfused, dehydrated, and emulsified muscles of rats manifesting toxicity with DDT.

[23] Doyère, M., *Annales d. Sciences Naturelles*, second series, 1840, **14**, 269.

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Diffusion of Sulfonamides and Penicillin into Fibrin.*

M. H. NATHANSON AND RUTH A. LIEBHOLD.
From the University of Southern California School of Medicine, and Research Laboratories, Cedars of Lebanon Hospital, Los Angeles.

Until the advent of penicillin, subacute bacterial endocarditis largely resisted all forms of therapy. The persistence of the disease is due to the survival of the bacteria in the valve locus. The organisms in the vegetations are separated from the blood stream by a layer of fibrin and it has been

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shown that antibacterial agents penetrate fibrin poorly or not at all. Friedman[1] studied the effect of 3 germicides, gentian violet, merthiolate and Vuzino-toxine camphorated on the *Streptococcus viridans* growing imbedded in a fibrin mass. He compared the effect with that occurring in ordinary broth cultures of the same organism and found that the fibrin mass had a striking retarding effect on the bactericidal action of these compounds. Friedman[2] also demonstrated the inability of sulfanilamide and sulfapyridine to eradicate a focus of streptococci growing in and protected by a fibrin-platelet mass. Duncan and Faulkner[3] were unable to demonstrate any appreciable penetration of sulfonamide compounds into blood clots in periods of 24 hours to 15 days. These observations suggest that failure of therapy in bacterial endocarditis may be due to an ineffective contact between chemotherapeutic agents and the bacteria on the valves as a result of impermeability of the fibrin barrier.

The sulfonamide compounds[4] and penicillin[5] inhibit the growth of *Streptococcus viridans* in the test tube. However, in bacterial endocarditis with the organisms lodged in the heart valve, penicillin has shown a striking therapeutic effect while the sulfonamides have at best exhibited only an occasional and usually temporary beneficial action. The present study is an attempt to determine whether the greater effectiveness of penicillin can be ascribed to a superior diffusion of this substance into fibrin. A comparison was made of the diffusibility into fibrin of the relatively ineffective sulfonamide compounds with that of penicillin.

Procedure. A modification of the cup assay method for penicillin was utilized. Agar plates were prepared using *Bacillus subtilis* as the test organism following the technic of Foster and Woodruff.[6] Fibrin plates were prepared in the following manner. Dried fraction I of human plasma† was reconstituted by the addition of saline, containing 10% glucose. Twenty ml portions were poured into petri dishes and 0.2 ml of a spore suspension of *B. subtilis* added to each plate.

The plates were shaken to permit an even distribution of the bacterial suspension. To each plate 0.2 ml of a solution of bovine thrombin was added. The thrombin was prepared by dissolving 5000 units in 5 ml of isotonic saline. The fibrin hardens within 2 minutes after the addition of the thrombin. Three penicillin assay cups were placed equidistantly on each agar and fibrin plate. The 3 cups were filled with the substance to be tested, permitting the reading of the results in triplicate. The plates were kept at room temperature as preliminary experiments showed that liquefaction occurred in the fibrin at incubator temperature. Measurements of the diameter of the zones of inhibition were made at 24-hour intervals.

Sulfonamide Compounds. Sulfathiazole and sulfadiazine were used in this study. Relatively dilute solutions of these compounds did not produce a definite inhibition in either the agar or fibrin plates. More concentrated solutions (M/25) of the sodium salts of these compounds showed definite zones of inhibition in the agar but there was no evidence of inhibition in the fibrin. To assay the possible effect of the alkalinity of these solutions, a control of disodium phosphate in M/25 solution, adjusted to the same pH as that of the sodium sulfonamides was utilized. One agar and one fibrin plate were set up with the 3 cups filled with the M/25 sodium sulfathiazole, another pair with M/25

1 Friedman, M., *J. Pharmacol. and Exp. Therap.*, 1938, **63**, 173.

2 Friedman, M., *Arch. Int. Med.*, 1941, **67**, 921.

3 Duncan, C. N., and Faulkner, J. M., *Am. J. Med. Sc.*, 1940, **200**, 492.

4 Orgain, E. S., and Poston, M. A., *Arch. Int. Med.*, 1942, **70**, 777.

5 Dawson, M. H., Hobby, G. L., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 101.

6 Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1944, **47**, 43.

† This plasma fraction was prepared under a contract between the Office of Scientific Research and Development, and Harvard University, from blood collected by the American Red Cross. This was supplied by the Department of Physical Chemistry, Harvard Medical School. In this fraction, 60% of the protein is fibrinogen and the remainder is albumin and globulin.

TABLE I.
Inhibition Zones Diameter in mm.
Diameter of zones of inhibition in mm on agar and fibrin plates produced by sodium sulfathiazole, sodium sulfadiazine, and disodium phosphate.

	M/25 Sodium-sulfathiazole		M/25 Sodium-sulfadiazine		M/25 Disodium-phosphate	
hr	Agar	Fibrin	Agar	Fibrin	Agar	Fibrin
24	0	0	0	0	0	0
48	12	0	0	0	0	0
72	15	0	14	0	0	0
96	18	0	18	0	0	0
120	18	0	18	0	0	0

TABLE II.
Diameter of Zones of Inhibition in mm.

Penicillin units/ml	0.5		1.0		2.0		4.0	
hr	Agar	Fibrin	Agar	Fibrin	Agar	Fibrin	Agar	Fibrin
24	9	9	9	9	11	9	11	10
48	10	9	11	9	12	9.5	15	12
72	11	9	12	10	12	10	18	12
96	12	10	13	12	14	13	18	14
120	14	10	16	12	16	14		
144	14	10	16	12	16	14		
168	14	10	16	12	16	14		

sodium sulfadiazine and the third with the control M/25 disodium phosphate. Readings were made at 24-hour intervals and the averages of the 3 readings taken. Table I shows the results of these experiments. It is evident that sodium sulfathiazole and sodium sulfadiazine diffuse into agar but show no reaction in fibrin. The control solution shows no inhibition in either the agar or fibrin plates indicating that the alkalinity of the sodium sulfonamides is not a factor in the reactions.

Penicillin. A number of experiments were carried out with varying concentrations of penicillin. A zone of inhibition appeared in both the agar and fibrin plates in 24 hours. Although the inhibition zones were somewhat larger in the agar as compared with fibrin plates, the difference was relatively slight, indicating a comparatively free diffusion of penicillin into fibrin. Table II shows the results of a typical experiment.

Discussion. These results indicate that penicillin diffuses freely into fibrin as compared with sulfonamide compounds. The conclusion seems justifiable that the greater effectiveness of penicillin in subacute bacterial endocarditis is at least in part due to this superior diffusibility. The fact that there is a relationship between the chemotherapeutic activity of a compound and its ability to permeate fibrin supports the concept of a fibrin barrier as the mechanism which permits the persistence of the infection on the valve. It is clear that in evaluating the efficiency of a drug in the treatment of bacterial endocarditis, the ability to penetrate fibrin requires important consideration.

A further implication of the present study relates to the use of anticoagulants in the treatment of subacute bacterial endocarditis. With the purpose of lessening the deposition of fibrin on the diseased valve, the administration of heparin has been used as an adjunct to antibacterial agents. Although some clinical reports suggest that heparin is beneficial, others indicate that the results of penicillin therapy are not improved by its use. The present experiments would tend to minimize the importance of heparin as it is shown that fibrin has little retarding effect on the activity of penicillin.

Summary and Conclusion. Using a modification of the cup assay method on agar and fibrin plates, there is no evidence of penetration of sulfathiazole and sulfadiazine into

fibrin. Penicillin diffuses almost as well into fibrin as into agar. It is suggested that the diffusibility of penicillin into fibrin is an important factor in the efficacy of this substance in the treatment of subacute bacterial endocarditis.

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Serum Levels After Repository Injections of Penicillin.

EDWIN M. ORY, CLARE WILCOX, AND MAXWELL FINLAND.

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

There is considerable interest in the development of methods for administering penicillin which are both simple and efficient. Oral administration would be the obvious solution were it not for the marked irregularity of absorption as well as the low efficiency of this method with the materials thus far available.[1-4] Various attempts have been made to maintain and prolong the effective concentrations of penicillin after parenteral administration with the hope of reducing the number of injections necessary. Most promising at the present time is the use of a repository injection of a relatively large dose in a medium from which absorption takes place slowly. Among the various combinations proposed, the mixtures of beeswax in peanut oil have been studied most extensively.[5-9] With this method effective serum levels can be maintained quite regularly over a 12-hour period and in most instances for 18 to 24 hours, but the concentrations after more than 12 hours following a dose of 300,000 units or less may be quite irregular or not measurable. While the beeswax-peanut oil mixtures have been used successfully in several clinics, their high melting point and the marked viscosity of the preparations make the injections of these materials technically difficult for most inexperienced persons. Substances which are more easily manipulated at ordinary room temperatures would be much more desirable.

Freund and Thomson[10] proposed the use of a simple, rapid technic of preparing water-in-oil emulsions of penicillin. The penicillin is put into solution in a small volume of saline and added to a mixture of a lanolin-like substance (Falba) and peanut oil and thoroughly emulsified before the injection. This method has been used by Cohn *et al.*[11] in the treatment of 52 cases of acute gonorrhea by the single injection of the emulsion containing 150,000 units of penicillin, with only 2 failures. The serum levels obtained in 3 subjects given the penicillin in this manner were somewhat better sustained than in 2 others in which the same amount of penicillin was given in saline. Similar or identical materials have been prepared commercially (Solvecillin, Pendil, Emulgen, etc.) and have the advantage that they flow readily at or

1 Free, A. H., Parker, R. F., and Biro, B. E., *Science*, 1945, **102**, 666.

2 Cutting, W. C., *et al.*, *J. A. M. A.*, 1945, **129**, 425.

3 Finland, M., Meads, M., and Ory, E. M., *J. A. M. A.*, 1945, **129**, 315.

4 McDermott, W., *et al.*, *Science*, 1946, **103**, 359.

5 Romansky, M. J., and Rittman, G. E., *Science*, 1944, **100**, 196.

6 Romansky, M. J., and Murphy, R. J., *J. A. M. A.*, 1945, **128**, 404.

7 Romansky, M. J., and Rittman, G. E., *New England J. Med.*, 1945, **233**, 577.

8 Leifer, W., Martin, S. P., and Kirby, W. M. M., *New England J. Med.*, 1945, **233**, 583.

9 Kirby, W. M. M., *et al.*, *J. A. M. A.*, 1945, **129**, 940.

10 Freund, J., and Thomson, K. J., *Science*, 1945, **101**, 468.

11 Cohn, A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 145.

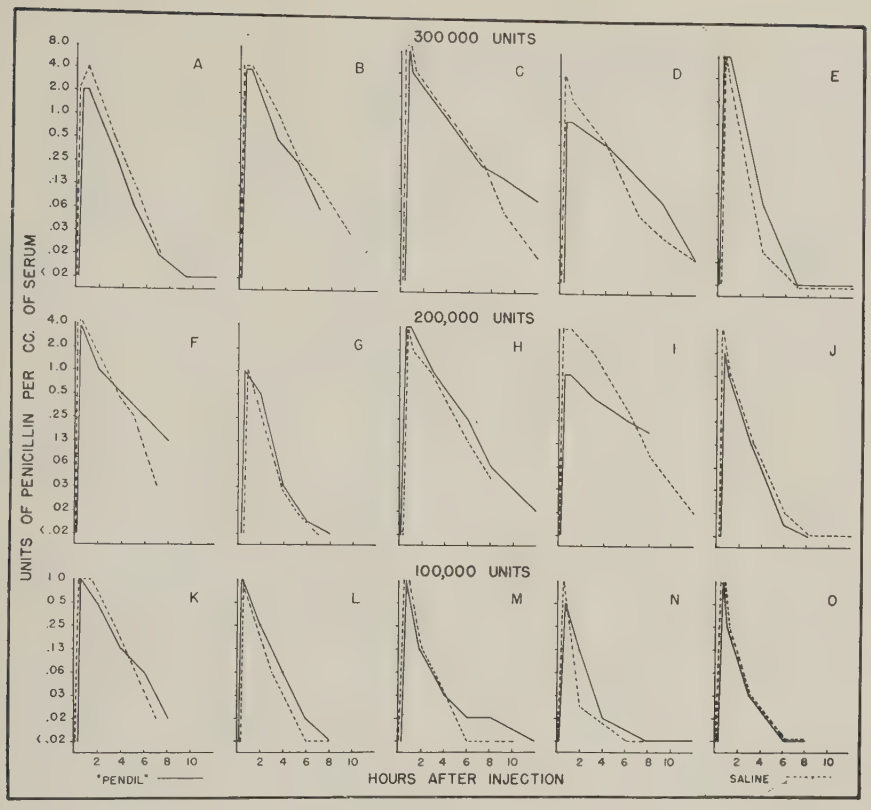

FIG. 1.

Serum penicillin concentrations in the same subjects after intramuscular injections of various doses in saline and in a water-in-oil emulsion.

slightly above room temperature and can therefore be easily manipulated. From that point of view, this type of mixture would be much preferable to the thicker beeswax mixtures, which readily solidify at the same temperatures.

In view of the fact that there may be considerable individual variations in the absorption of penicillin even after intramuscular injections of the same dose, it seemed essential to compare serum levels obtained from any given dose in the repository injection with those obtained with saline solutions in the same individuals.

Intramuscular doses of 100,000, 200,000 and 300,000 units, each in a volume of 4.5 cc were used. The subjects were ward patients who had not recently received any chemo- or antibiotic therapy. About one-half of the subjects were given a single dose of penicillin in saline and 3 or more days later the same amount of penicillin was given in water-in-oil emulsion* as advocated by Freund and Thomson.[10] The other subjects received the doses in the reverse order. Blood was drawn at intervals after the injection and the concentration of penicillin in the serum determined by a slight modification of the serial dilution method of Rammelkamp.[12] The minimum concentration detectable by this method was 0.0156 unit per cc of serum.

The results are shown in Fig. 1. There were slight variations in the maximum concentrations obtained and somewhat greater individual variations in the maintenance of serum levels after the same dose. The larger doses gave higher and more prolonged levels in either menstruum. There was no constant

* "Pendil," generously supplied by Endo Products, Inc., through the courtesy of Dr. Samuel M. Gordon.

12 Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

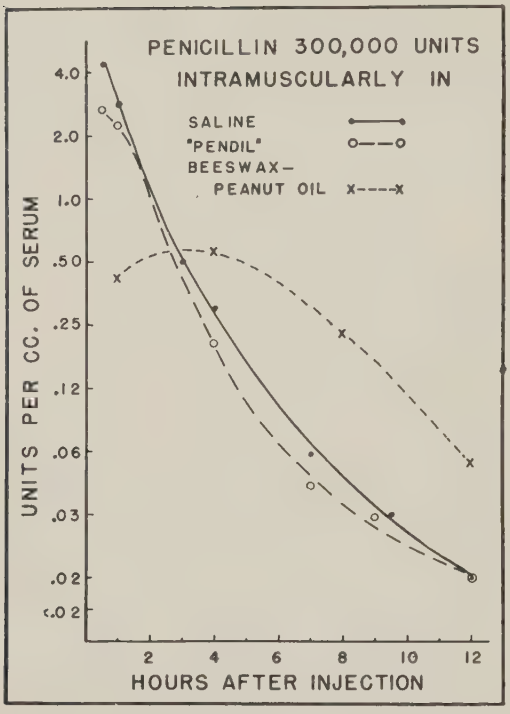

FIG. 2.
Serum penicillin concentrations after intramuscular injections of 300,000 units of penicillin in 3 vehicles.

or striking difference between the results obtained in the same subject after the same dose given in the 2 vehicles.

In Fig. 2 are shown the mean concentrations obtained over a 12-hour period after intramuscular injections of 300,000 units of penicillin given in saline, in Pendil, and in 4.8% beeswax in peanut oil (Delacillin, Squibb), the latter being contained in a volume of 1 cc. Each curve is based upon the results obtained in 6 subjects. It is seen that the maximum concentration obtained with the beeswax-peanut oil mixture was appreciably lower, but the levels were much better sustained than with the same dose in saline or in Pendil.

Conclusions. When tested in the same subject, the serum concentrations obtained after a single injection of penicillin in a water-in-oil emulsion were not superior to those obtained with the same dose given in the same volume of saline, and the levels were not better sustained. A dose of 300,000 units in 1 cc of 4.8% beeswax in peanut oil gave lower maximum concentrations, but the serum levels were better sustained over a 12-hour period.

15382 P

Suppression of Circulating Antibodies in Pyridoxin Deficiency.

HERBERT C. STOERK AND HERMAN N. EISEN.

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City.

There has recently accumulated evidence to indicate that there may be an important relationship between lymphocytes and antibodies.[1,2,3] Data suggesting that pyridoxin may be a factor essential for the maintenance of lymphoid tissue have been reported previously.[4,5,6] It therefore seemed of interest to study the effect of pyridoxin deficiency on circulating antibodies.

Methods. Twenty-four male albino rats of the Sherman strain, close to 4 weeks of age, were divided into 3 groups of litter mates.

The animals in the first group were weaned

1 Ehrich, W. F., and Harris, T. N., *Science*, 1945, **101**, 28.

2 Daugherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 295.

3 Daugherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 135.

4 Stoerk, H. C., and Zucker, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 151.

5 Stoerk, H. C., *Fed. Proc.*, 1946, **5**, 227.

6 Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., in press.

TABLE I.
Effect of Pyridoxin Deficiency upon Serum Antibodies.

Group	Experiment	No. of rats	Average Initial body wt, g	Final body wt, g	Thymus wt, g	Agglutinin titers Avg	Agglutinin titers Range	Hemolysin titers Avg	Hemolysin titers Range
I	Pyridoxin deficient	9	47	83	.047	1:0.4	0-1:4	1:13	0-1:80
II	Paired weighed	7	46	78	.182	1:64	1:32-1:128	1:412	1:160-1:640
III	Control	8	45	160	.395	1:36	1:10-1:80	1:68	1:20-1:160

on a diet deficient in pyridoxin but otherwise adequate.[4] Daily weights were recorded in these animals. Restricted amounts of the complete diet were fed to the second group in order to duplicate the growth retardation produced by pyridoxin deficiency (paired weighing). The third group received the complete diet *ad libitum*. During the fifth week of the dietary experiment all animals were immunized against washed sheep erythrocytes. Three intraperitoneal injections of 0.5 ml of a 5% suspension of red cells were administered on alternate days. Five days after the last injection the animals were exsanguinated under sterile precautions. Hemagglutinin and hemolysin determinations were carried out on individual sera by the usual method of serum dilution in 2-fold steps. The titer endpoints adopted were 3 plus for agglutinins and 2 plus for hemolysins.

Immediately after the bleeding the thymus and other organs were weighed and prepared for histological study.

Results. The data are summarized in Table I. It is apparent that there occurred growth retardation in Groups I and II, with very low quantities of circulating antibodies in the pyridoxin-deficient animals. Although the scatter of the antibody values is great in both control groups, the difference between these and the deficient group is most striking. Of the 9 pyridoxin-deficient animals 6 showed no measureable agglutinins or hemolysins and 3 had only very low titers. In the 2 control groups all 16 animals had measurable antibody titers, and 13 of these were comparatively high.

The extreme degree of lymphoid atrophy was apparent not only from the weight of the thymus, but also from their morphology. In the thymi and lymph nodes of the deficient animals there was observed a pronounced reduction in the number of lymphocytes.

Discussion. From the data presented it is apparent that animals fed a diet deficient in pyridoxin and then immunized exhibited little or no circulating antibodies. The immunization was begun at a time when lymphoid atrophy was advanced. From the data on hand, however, it cannot be decided that the suppression of the serum antibodies was a consequence of the lymphoid atrophy: It is possible that both alterations occur independently in pyridoxin deficiency. Further study of this point is now in progress.

Summary. Male albino rats immunized in a state of pyridoxin deficiency developed antibody levels in the serum far below those of inanition controls (paired weighed) and full controls.

We wish to express our thanks to Dr. M. Mayer for giving generously of his time and advice on the immunological procedure.

15383

Effects of Calcium Deficiency and Pyridoxin Deficiency on Thymic Atrophy (Accidental Involution).

Herbert C. Stoerk.

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City.

In a variety of experimental conditions atrophic thymus glands have been described by many observers. In many of the cases this atrophy has been attributed to the variable introduced by the experiment. The specificity of the observed effect, however, was not ascertained by sufficient controls.

In the following it has been attempted:

1. To establish the approximate norm for thymus size in albino rats raised under con-

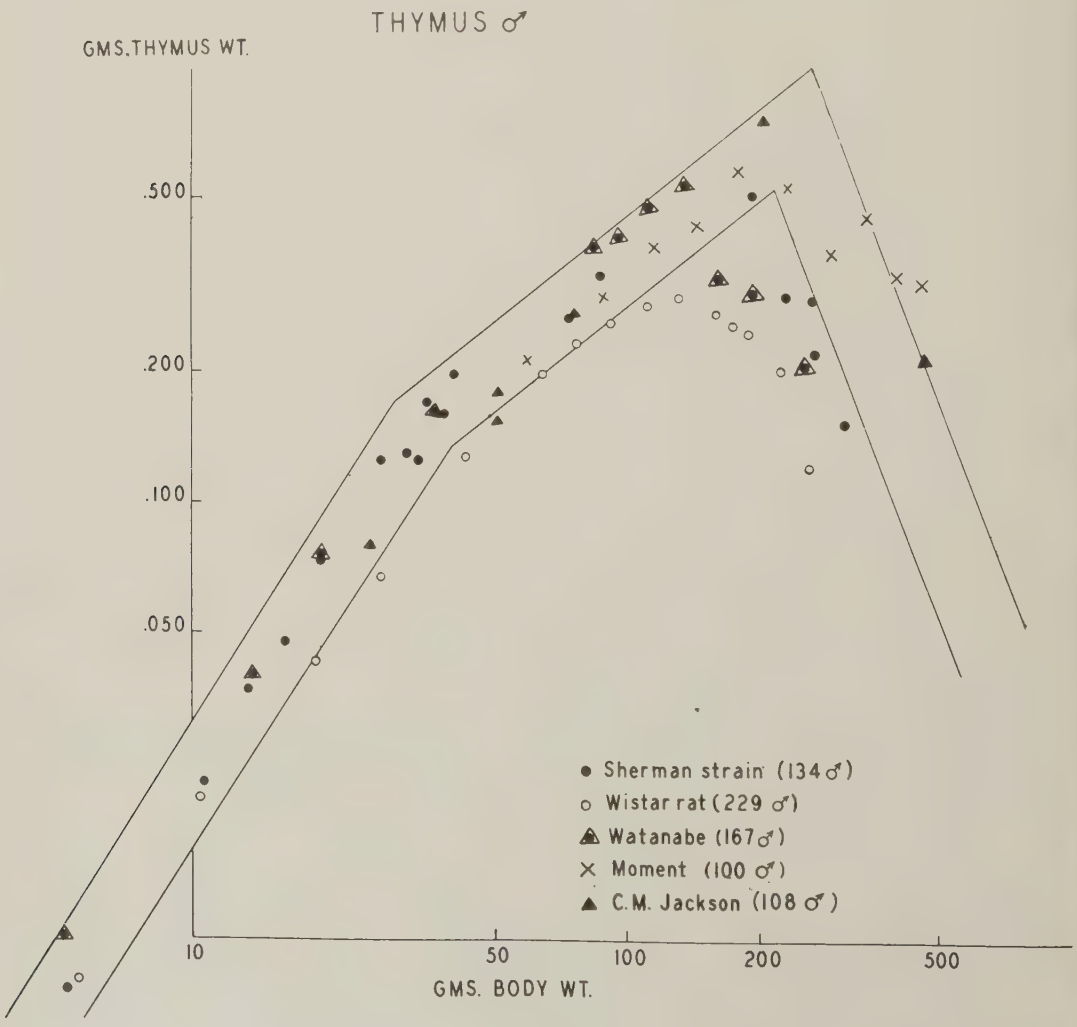

Chart I.
Log./Log. plot of thymus weight on body weight in male albino rats.

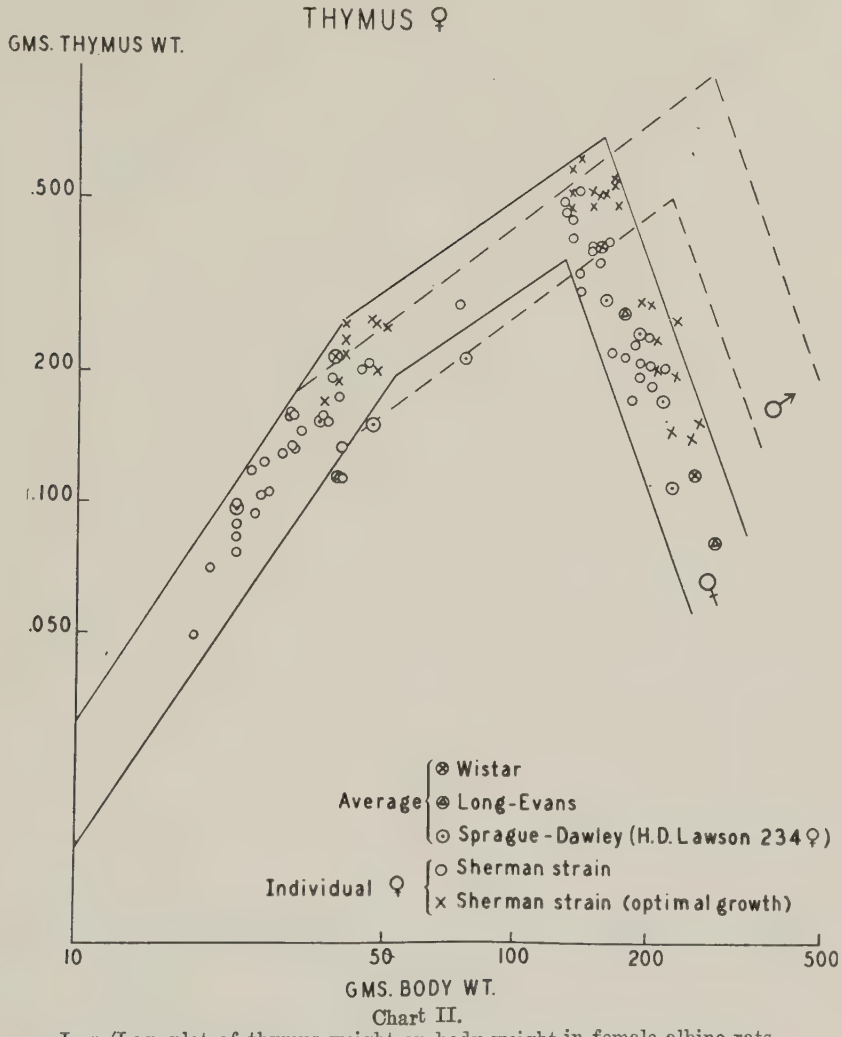

Chart II.

Log./Log. plot of thymus weight on body weight in female albino rats.

ditions probably close to optimal.

2. To study the extent of thymic atrophy (accidental involution) produced by closely controlled experimental conditions.

All of the experimental conditions induced, represented adverse bodily conditions and were associated with growth, retardation or body weight loss. The extent of the harmful stimulus was judged by its effect on body weight.

In all conditions where effects on thymic tissue were observed the lymphoid tissue appeared similarly affected. This was observed from the histology of lymph nodes and spleen.

Several attempts have been made in the past to determine the norm for thymus size in the rat over a range of age or body weight.[1-6] Only part of the data, however, were collected recently enough to take into account the newer knowledge of the dietary requirements in this species.

1 Donaldson, H. H., *The Rat*, 1924, Mem. Wistar Inst.

2 Jackson, C. M., *Anat. Rec.*, 1937, **68**, 371.

3 Watanabe, T., *Trans. Jap. Path. Soc.*, 1929, **17**, 332.

4 Moment, G. B., *J. Exp. Zool.*, 1933, **65**, 359.

5 Lavson, H. D., *et al.*, *Endocrinology*, 1942, **31**, 129.

6 Stoerk, H. C., *Endocrinology*, 1944, **34**, 329.

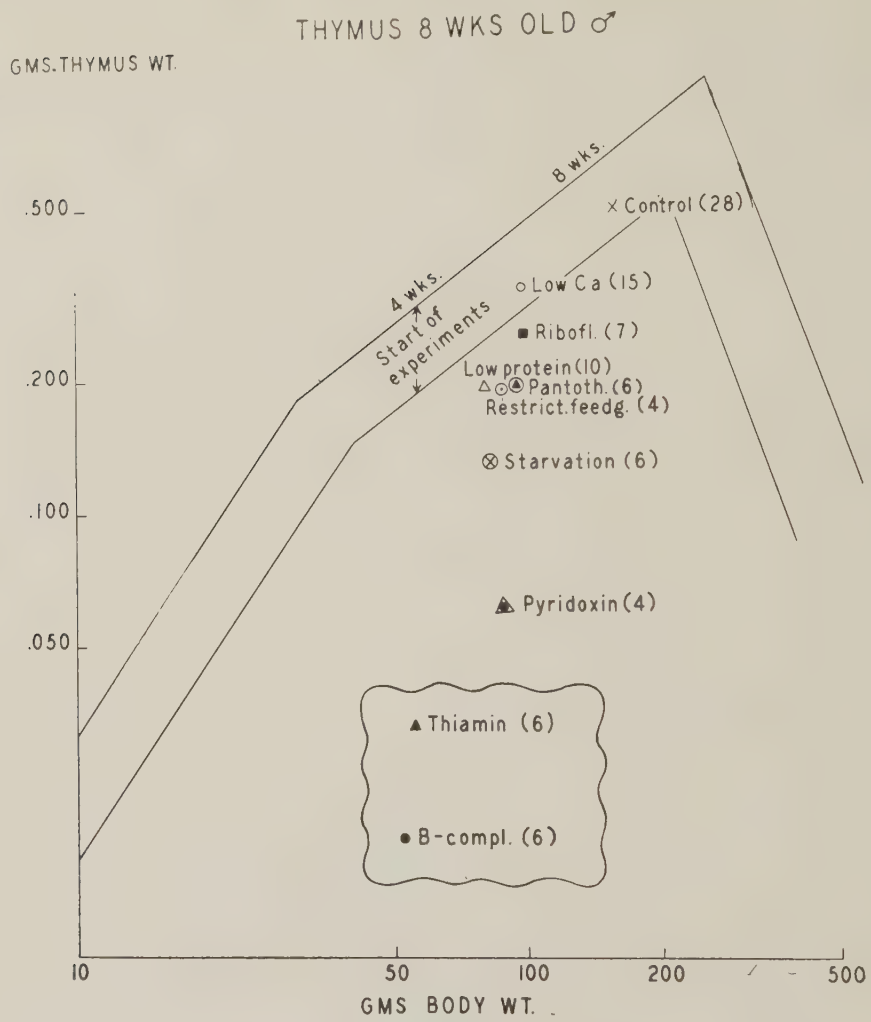

Chart III.

Range of thymus weights obtained from animals growing optimally (Chart I), compared with experimental data.

Recently Zucker and Zucker[7] have shown that in albino rats fed an adequate stock ration, there exists a simple body weight time relation. Their growth formula applied not only to large numbers of rats of their own colony but also to data of others who employed complete diets. This body weight time relationship, obviously cannot be obtained when factors other than dietary interfere with normal growth. Such a factor exists in most rat colonies in form of a large variety of known and unknown diseases.

In the early stages of this investigation a relatively large number of thymus weights was recorded from animals of our stock colony (Sherman strain) kept on a complete ration (Rockland rat diet). Growth records as far as available showed optimal growth only in isolated cases. Later care was taken to improve the living conditions of the animals and to follow their growth regularly. It became evident then that animals showing optimal growth had larger thymus glands than undersized rats. The cause of the growth retardation in the undersized animals was not known but the failure to obtain optimal

[7] Zucker, L., and Zucker, T. F., *J. Gen. Physiol.*, 1942, **25**, 445.

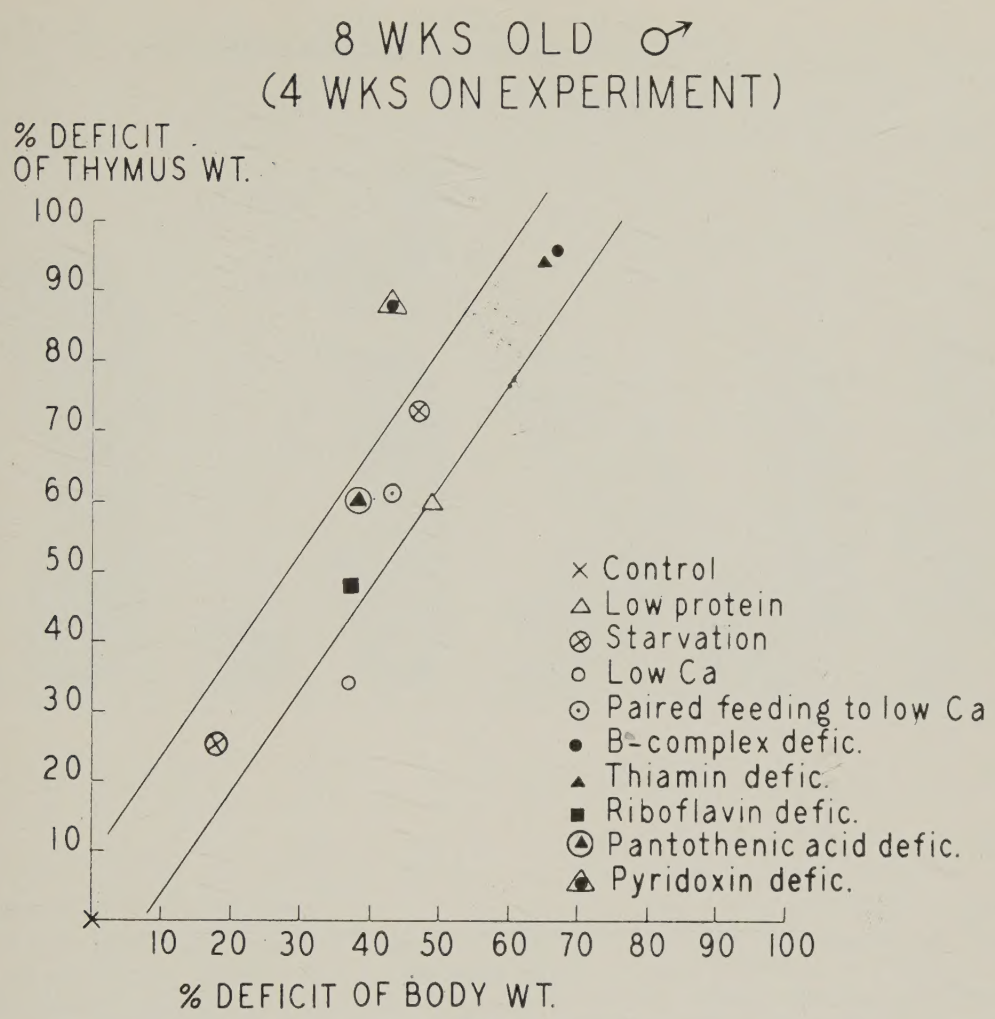

Chart IV.
Relationship between body weight deficit and thymus weight deficit.

growth on a complete ration was considered as an abnormality and thymus weights in such animals were excluded from the use as controls.

The accepted norm of thymus weight was obtained with any of several complete diets, provided that growth was optimal. None of these diets had an apparent effect *per se* on thymus weight.

Chart I presents a log/log plot of thymus weight on body weight of male albinos of the Sherman strain from our stock colony as compared with other data from the literature. The band includes the range of our data obtained from 115 animals that grew either optimally or recovered from previous growth disturbance. As reported previously an identical range of thymus weights on body weight was obtained in 56 male rats, castrated or adrenalectomized 3-4 weeks before killing.[6] Jackson and Moment who employed animals growing close to optimally have obtained thymus weights as high as those found in our controls, or even somewhat higher. Fair agreement of all data is evident in the earlier part of the rat's life. The thymus weights from about 100 g of body weight on in Donaldson's (Wistar rat) and Watanabe's data and in our own earlier observations are sig-

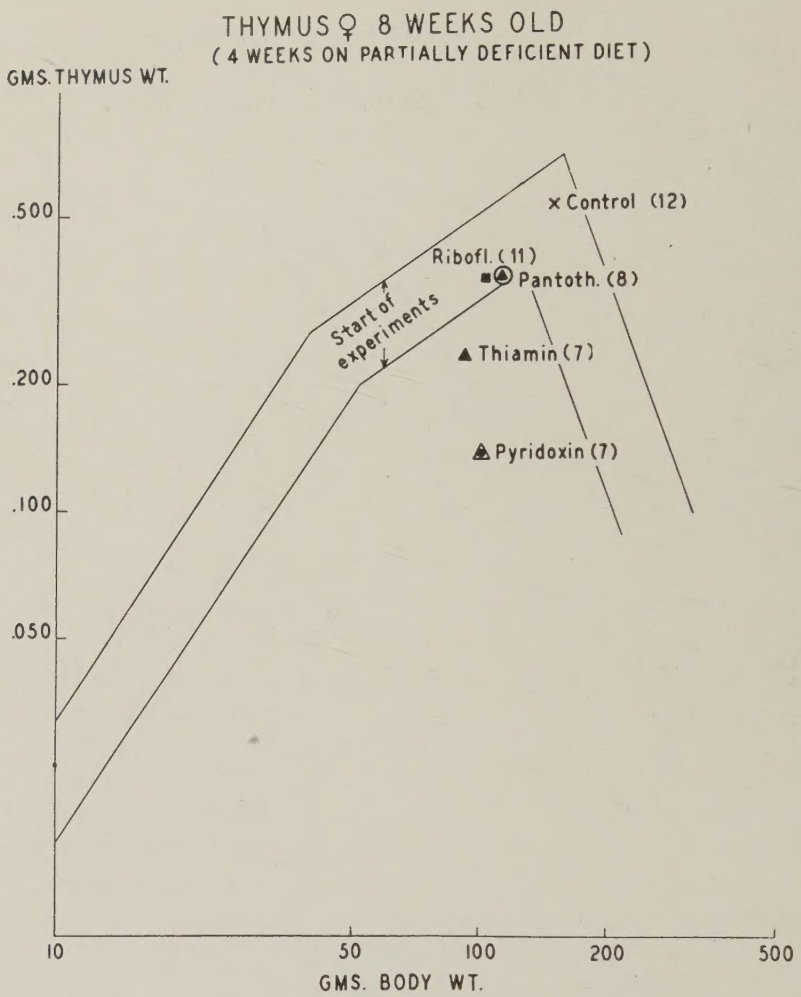

Chart V.
Range of data in Chart II compared with experimental data.[9]

nificantly lower than the accepted norm. In these 3 groups the growth of the animals was below optimal.

Chart II presents a similar plot illustrating thymus growth on body weight in the female albino.

Chart III gives a comparison of experimental data in the 8-week-old male. The band again represents the range of the accepted norm. A number of adverse dietary conditions, mostly produced by the omission of a single substance, from the diet, have been induced for a period of 4 weeks.* It is seen that in most of these conditions the thymus ceased to grow or was slightly atrophic when body growth was retarded. In deficiency of B-complex and in thiamin deficiency growth retardation was marked and was followed in the latter part of the experiment by body weight loss. In these 2 cases, thymic atrophy was extreme. Advanced thymic atrophy without marked effect on body weight was observed only in pyridoxin deficiency. Similar findings in the female rat have been reported previously.[8] No retarda-

* The basic diet was the same as used by Berg, B. N., and Zucker, T. F., Abstracts 107th A. C. S. Meeting, Cleveland, O., April, 1944. The thymi of the animals deficient in single B-factors were kindly given to us by the above authors.

8 Stoerk, H. C., and Zucker, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 151.

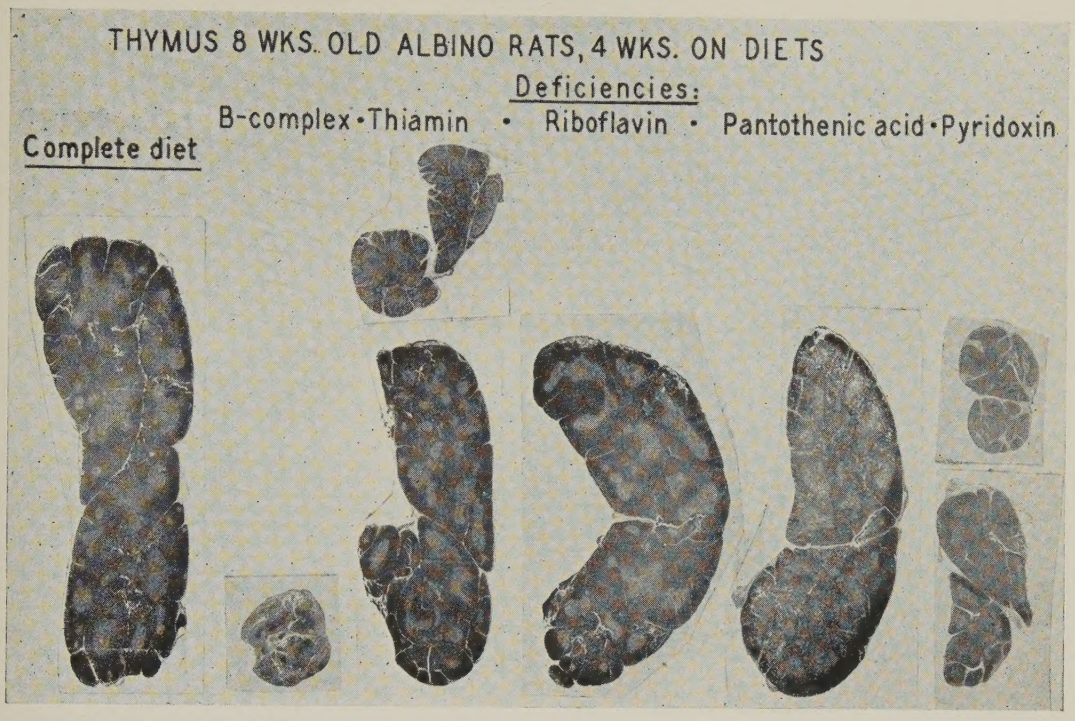

Fig. 1.
The sections of thymus are representative of the glands closest to the average weight in the respective group, except for those in thiamin and pyridoxin deficiency where the smallest and the largest gland in the group have been photographed. (Actual maximal diameter of control gland 10 mm).

tion of thymus growth was evident when body growth retardation and even weight loss was produced by calcium deficiency.

The above data are compared in Chart IV where the thymus weight deficit in percent below that of the controls is plotted against the body weight deficit calculated on the same basis. Roughly most values obtained fit a straight line, the slope of which is about 1.5. Marked deviations from this approximate straight line relationship are evident in 2 cases. In pyridoxin deficiency the thymus weight deficit in relation to body weight deficit was unduly high; in Ca deficiency this ratio was lower than expected. The latter finding is analogous to what is observed on proper comparison, in gonadal and adrenal insufficiency.[6] This occurrence in Ca deficiency is apparently not related to hypocalcemia since parathyroidectomized rats did not show a discrepancy between body weight and thymus weight deficit.[9]

The specific effect of pyridoxine deficiency in producing thymic atrophy is also brought out from the data plotted in Chart V. In this experiment partial deficiencies of single B-factors are compared in female rats. Body growth was almost identical in all groups except that the animals receiving low thiamin lost weight towards the end of the experiment. Actual thymic atrophy was found only in the animals partially deficient in pyridoxin.

In Fig. 1 the histology of glands, of female rats representative of their group, is compared in animals deficient in B-complex, and deficient in single B-factors. It is seen that the marked effect of pyridoxin deficiency on the thymus is also obvious from the morphology. Lymph nodes in these animals were likewise greatly depleted of lymphocytes.

Fig. 2 shows a microphotograph of an atrophic thymus gland representative of those of 10 mice fed a diet deficient in pyridoxin and injected with .2 mg Desoxypyridoxin (kindly supplied by Merck & Co.) 2 times

[9] Unpublished observations.

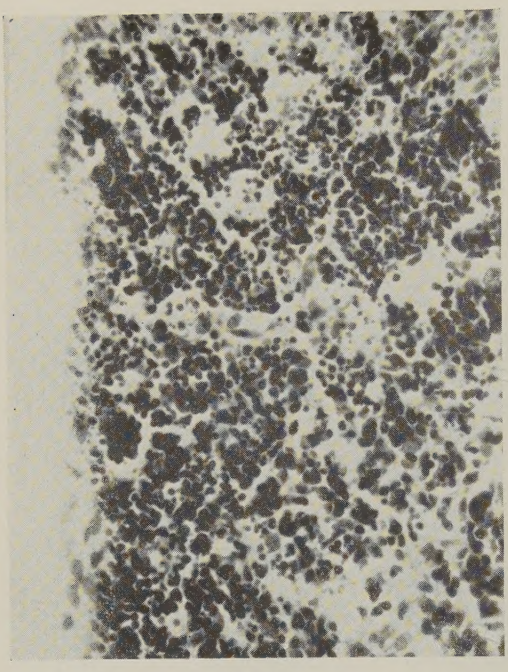

Fig. 2.
Thymus of mouse injected with desoxypyridoxin. Note pycnosis and fragmentation of lymphocytes. The cortical tissue was markedly reduced in amount.

daily over 3 days. The thymi of the injected animals weighed about 50% less than those of non-injected controls. Further studies are necessary to ascertain that no toxic effects are responsible for this change.

The apparently specific effect of even relatively mild pyridoxin deficiency in producing lymphoid atrophy suggests the possibility that pyridoxin may be essential for the maintenance of lymphocytes. This possibility has become of increased interest with the recent finding that a desoxypyridoxin is a powerful inhibitor of B-6.[10] This preparation may therefore perhaps be helpful in the treatment of tumors of the lymphoid tissue. The role recently attributed to lymphocytes in relation to antibodies, and the finding that pyridoxin deficiency suppresses circulating antibodies[11] suggests the possibility that desoxypyridoxin may be used in order to interfere with undesired antibody formation. Investigations in these directions are being carried out.

Summary. In male albino rats (8 weeks of age) exposed to a number of adverse dietary conditions, an approximately linear relationship was found between the amount of body weight deficit and the thymus weight deficit. There were 2 exceptions from this apparent rule: (1) in pyridoxin deficiency, the amount of thymus weight deficit was much greater than expected from the obtained body weight deficit; (2) in calcium deficiency an opposite effect was observed. Here the thymi of undersized animals were of the same weight as those of younger, normal animals of the same body weight.

[10] Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 125.

[11] Stoerk, H. C., and Eisen, H. N., PROC. SOC. EXP. BIOL. AND MED., 1946, in press.